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ACTIVE IMMUNIZATION OF BIRDS AGAINST MALARIA

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It has been reported that ducks[1] and monkeys[2] can be immunized against blood-induced malaria by inoculating with killed parasites in a water-in-oil emulsion with killed tubercle bacilli. The ducks were immunized with whole parasitized erythrocytes. In the present study this method was used to immunize ducks and chickens against *Plasmodium lophurae* and chickens against *Plasmodium gallinaceum*. This study was carried out to ascertain the role of iso-antibodies to erythrocytes in immunities elicited with such vaccines.

MATERIALS AND METHODS

The strain of *P. lophurae* is the strain isolated and described by Dr. L. T. Coggeshall.[3] It was obtained from Dr. E. K. Marshall for immunity studies in 1944 and 1945, was repeatedly transferred by blood passage in chickens, was transferred to ducklings in 1948 and maintained in them throughout the present experiments. The strain of *P. gallinaceum* is designated 8A by the American Society of Parasitologists. The course of infection was observed by daily counts of parasites per 10,000 erythrocytes done by examining stained thin smears of blood with an oil-immersion microscope. With counts of 5000 or more, the parasites in 100 erythrocytes were counted; with counts of about 500, the parasites in 10 microscopic fields containing about 100 erythrocytes per field were counted; with counts of 50 or less, the parasites in 50 fields containing about 100 erythrocytes were counted. According to the formula of Gingrich[4] the probable error of a count of 50 parasites per 10,000 erythrocytes is about 10%. The lower counts are much less accurate.

The hosts were white Peking ducks and chickens of mixed breed. Infection was induced by the intravenous injection of parasitized blood cells not more than 15 minutes after they were drawn from the infected donor.

To prepare vaccine against *P. lophurae* from infected ducks or chickens or against *P. gallinaceum* from chickens, parasites were obtained by bleeding birds having approximately one parasite (or more) per erythrocyte. As an anticoagulant, one part of 4% sodium citrate in 0.85% sodium chloride was used per 9 parts of blood. Parasitized erythrocytes were washed 5 times by centrifuging and resuspending in isotonic saline and were left overnight in 0.1% formaldehyde saline. They were then washed two or three more times in saline. The parasitized cells, suitably diluted with saline, were mixed in a Waring blendor with the following materials in nearly the same proportions as described by Thomson et al.[1] For example:

180×10^9 parasites (120×10^9 erythrocytes) in	52.8 ml
"Falba"*	26.4 ml
"Bayol F"†	52.8 ml
Killed dried tubercle bacilli‡	24 mg
Total volume	132.0 ml

The proportion of aqueous suspension to Bayol, Falba and tubercle bacilli was the same in all vaccines. Each control vaccine was made up simi-

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1. Thomson, K. H., Freund, J., Sommer, H. E. and Walter, A. B. 1947, Am. J. Trop. Med. **27**: 79–105.
2. Freund, J., Thomson, K. J., Sommer, H. E., Walter, A. B. and Pisani, T. N. 1948, Am. J. Trop. Med. **28**: 1–27.
3. Coggeshall, L. T. 1938, Am. J. Hyg. **27**: 615–618.
4. Gingrich, W. D. 1932, J. Prev. Med. **6**: 197–246.

* Manufactured by Pfaltz and Bauer, Empire State Bldg., New York City.

† Kindly furnished by Penola Inc., Linden, N. J.

‡ Kindly given by Dr. Jules Freund.

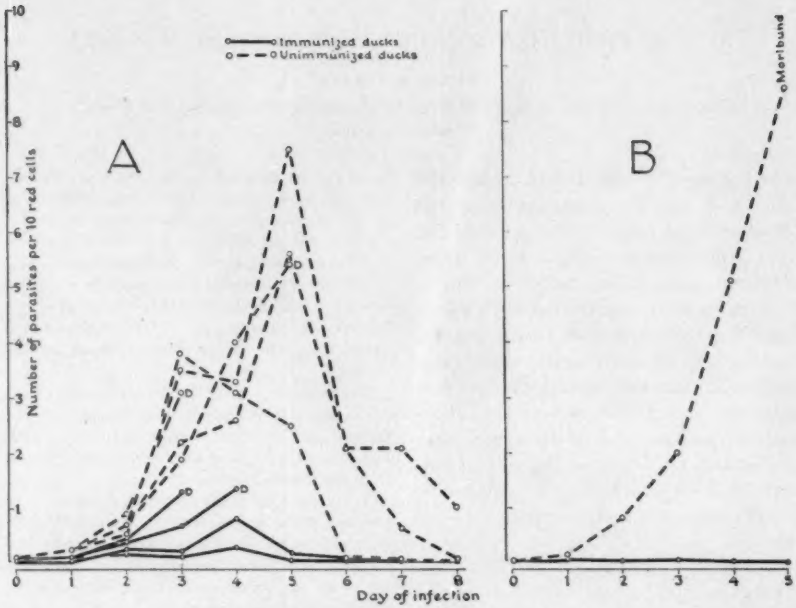

FIG. 1A and 1B.—Parasite counts of *P. lophurae* in large ducks immunized by a vaccine of killed *P. lophurae* with adjuvants (solid lines) and in controls (dash lines). D = dead. Note the immunization.

larly except that parasites were omitted. The vaccine was used on the day it was prepared. The water-in-oil emulsion and the killed tubercle bacilli will be referred to hereafter as adjuvants.

In the experiment shown in figure 1A, immunized birds received vaccine prepared with parasitized erythrocytes from one duck, and control birds received vaccine prepared on the same day with normal erythrocytes obtained earlier from the same duck. Vaccines for the second immunizing injection were prepared in the same manner from a second duck. To ascertain whether the vaccines were infectious, two or three ml of blood were obtained from each immunized bird four days after the first immunizing injection and injected intravenously into a day-old duckling. No infection was detected in any of the ducklings. After the second immunizing injection, blood of each immunized bird was examined daily for 10 days, and no parasites were found.

EXPERIMENTAL RESULTS

Homologous immunization of ducks against P. lophurae.—White Peking ducks weighting initially 1.7 to 2.3 kg were used in 4 groups of 6 birds. In the first group, each duck was inoculated twice, at an interval of 6 weeks, with the following: 11 ml of vaccine, containing 15×10^9 parasites in adjuvants and injected into 6 sites in the pectoral muscles. A second group was inoculated similarly with vaccine prepared with unparasitized duck erythrocytes, a third group was given vaccine prepared with human erythrocytes (group A) and a fourth group was left untreated.

All surviving birds were challenged, 16 weeks after the first injection of vaccine, by an intravenous injection of 10^9 trophozoites of *P. lophurae* per kg duck. Blood parasite counts of all the challenged birds were made each morning. As shown in figure 1A, parasite-vaccinated birds had much lower blood

parasite counts than unvaccinated birds. Mortality in the two groups was similar. Parasite counts of birds immunized with normal duck or human erythrocytes were as high as those of unimmunized birds and are not included in the figure. A similar experiment with one parasite-immunized and one normal untreated duck resulted in an even more marked protection as shown in figure 1B.

Other attempts to immunize ducks using shorter periods of immunization.—Immunization for a shorter period with approximately half the number of parasites used for immunization in the preceding section gave no immunity. Ten ducks weighing 2100 and 2800 g were immunized by a single injection of a freshly prepared vaccine of 16×10^9 *P. lophurae* in 10 ml of adjuvants, injected into 6 sites in the pectoral muscles. Eight weeks later these and 5 control birds were infected intravenously with 1.1×10^9 parasites/kg duck. Blood parasite counts were similar in the immunized and control birds, but 4 immunized birds died and all 5 controls survived.

Two attempts to immunize small ducklings with vaccines which had been stored at about 6 C for periods varying from two days to 6 months were unsuccessful, although they received large amounts of antigen. Thus, 7 ducklings weighing 400 to 700 g were immunized by 4 intramuscular injections at 2-week intervals with 16×10^9 parasites in 8 ml of the usual adjuvant emulsion. Other groups of ducklings were immunized with similar numbers of parasitized erythrocytes in isotonic saline, with normal duck erythrocytes in saline, and with normal human A erythrocytes in saline. Eight weeks after the first immunizing injections, these ducklings and 10 controls were infected intravenously with 2.5×10^8 *P. lophurae* per kg duckling. At this time the birds weighed 1250 to 2250 g. Parasite counts did not reveal any immunity in the 4 groups of immunized birds.

Seven ducklings, weighing approximately 125 g, were immunized for a period of 4 weeks with 4 injections, a total of 20 ml of vaccine in adjuvants, containing 4×10^9 parasites per ml. Seven ducklings were immunized similarly with normal duck red blood cells.

TABLE 1.—*Parasite count per 10,000 red cells after first and second challenging dose of* P. lophurae *in chickens immunized as described in the text.*

Day	First challenge				
	Immunized		Controls		
Chicken:	1	2	3	4	5
1	0	0	0	0	0
2	0	0	0	0	0
3	0	0	4	0	0
4	0	0	6	2	0
5	0	0	28	2	0
6	0	0	20	8	2
7	0	2	2	36	0
8	0	2	0	2	0
9	0	0	0	12	0
10	0	0	0	0	0

	Second challenge						
	Immunized					Controls	
Chicken:	1	2	3	4	5	6	7
1	0	0	220	120	140	140	230
2	0	0	0	0	2	70	360
3	0	0	0	0	0	260	1120
5	0	0	0	0	0	2	150
6	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0

Seven weeks after the first immunizing injection, these and 8 controls were infected intravenously with 500×10^6 parasites. Parasite counts showed no immunity. Thomson et al[1] were also unable to induce immunity in young ducklings.

Homologous immunization of chickens against P. lophurae *and heterologous immunization of chickens against* P. gallinaceum.—Two chickens weighing about 2.5 kg were immunized with vaccine prepared as previously described from chickens infected with *P. lophurae*. Each bird was injected intramuscularly in 6 sites with a total of 10 ml of vaccine containing 13×10^9 parasites in

14.4×10^9 chicken red cells. These birds and three controls were challenged 8 weeks after the one immunizing inoculation by an intravenous injection of 4.8×10^{14} trophozoites of *P. lophurae*. As shown in table 1, there appeared to be some immunity. Five of these 6 birds were challenged again 10 weeks after the immunizing inoculation with 6.6×10^9 *P. lophurae* given intravenously. A

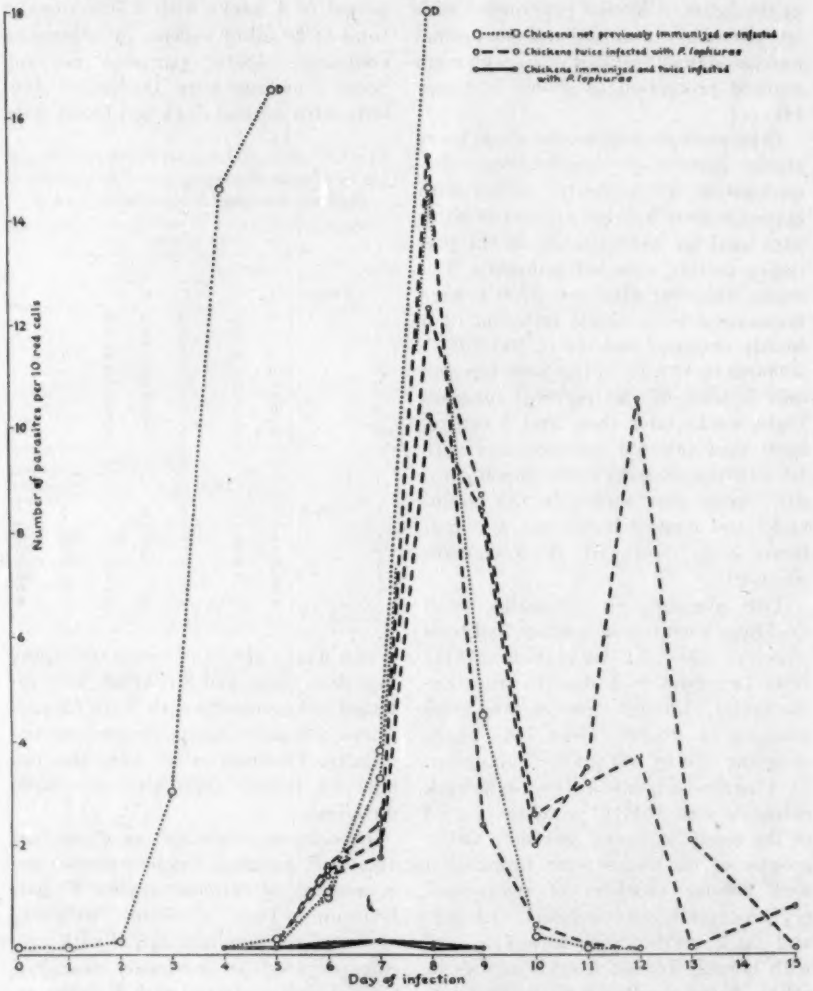

FIG. 2.—Parasite counts of *P. gallinaceum* in large chickens (1) immunized by a vaccine of killed *P. lophurae* with adjuvants and infected twice with *P. lophurae*, (2) infected twice with *P. lophurae* and (3) untreated controls. Note the marked heterologous immunity conferred on group 1 as compared to those of groups 2 and 3.

stronger resistance occurred in the immunized birds than in the controls. Twelve weeks after the immunizing injection, the same 5 birds and 3 controls of similar weight (2.1 to 2.7 kg) were challenged by an intravenous injection of 114×10^6 *P. gallinaceum*. As shown in figure 2, the birds immunized with the vaccine and twice infected with *P. lophurae* strongly resisted infection with *P. gallinaceum*, while the birds which had been infected twice but not immunized with the vaccine had little more resistance than normal chickens. These results are of particular interest, because infection followed by 4 superinfections with blood-induced *P. lophurae* results in only a transitory immunity to *P. gallinaceum*, as reported by W. H. and L. G. Taliaferro.[5]

Partial immunization of chickens against P. gallinaceum *with normal chicken erythrocytes.*—Five chickens weighing at first 0.5 to 1.2 kg were immunized by 5 weekly intramuscular injections of 20×10^9 normal chicken red cells in 10 ml saline suspensions. The red cells were all obtained from the same donor. Six chickens weighing 0.8 to 1.2 kg were immunized similarly, but received suspensions of their own red cells collected prior to immunization. Two weeks after the last immunizing injection, these and 5 control birds were given an intravenous injection of chicken blood containing 42×10^7 *P. gallinaceum*/kg recipient. Some immunity appeared in all the birds immunized with normal red cells from another chicken, as shown by a slightly lower peak (1800 to 15,200 against 12,700 to 17,000), a slightly longer prepatent period (4 to 8 against 1 to 3 days) and a greater recovery rate (4 of 5 against 2 of 5) in the test birds as contrasted with the control birds. Inconsistent results were obtained in the birds immunized with their own erythrocytes—a few showed some immunity.

A second experiment involved extra control birds. Six chickens weighing 0.2 to 0.3 kg were immunized by a series of 5 weekly intramuscular injections of 8×10^9 normal chicken erythrocytes, obtained from several donors and suspended in normal saline to make 4 ml per injection. Groups of 6 chickens were also immunized with (1) parasitized chicken erythrocytes (1 to 1.5/red cell) obtained from several other donors and suspended in saline, (2) parasitized erythrocytes (*P. gallinaceum*) in 4 ml adjuvants and (3) normal human erythrocytes in saline. These 4 groups and 6 normal chickens were challenged two weeks after the last immunizing injections by the intravenous administration of 20×10^6 *P. gallinaceum*/kg chicken. At this time the chickens weighed 0.5 to 1.1 kg. As shown in figure 3, the parasitemia did not increase as soon in the birds immunized with normal chicken red cells. This slight delay was not seen in birds immunized with human A erythrocytes or erythrocytes parasitized by *P. gallinaceum* whether in saline or with adjuvants. For clarity, data concerning these latter birds are omitted from the figure.

Other attempts to immunize chickens against P. gallinaceum.—Four chickens weighing 2.3 to 2.8 kg were inoculated, in 8 sites in the pectoral muscle, with a total of 16×10^9 formalin-killed parasitized *P. lophurae* duck red blood cells, one parasite per cell, in 10 ml of water-in-oil emulsion with killed tubercle bacilli. Four more were inoculated on the same day with an emulsion of normal erythrocytes from the same duck. When these and four normal chickens were challenged 7 weeks later by the intravenous injection of 2.1×10^9 *P. gallinaceum*, no immunity was seen;

5. Taliaferro, W. H. and Taliaferro, L. G. 1945, J. Infect. Dis. 77: 224–248.

mortality and parasite counts were high in all groups.

Four chickens weighing 2.25 to 2.75 kg were inoculated in 8 sites in the pectoral muscle with 15×10^9 formalin-killed *P. gallinaceum* in 10 ml of adjuvants, and 4 controls were similarly inoculated with an emulsion of normal erythrocytes of the chicken from whose blood the vaccine of *P. gallinaceum* was prepared. Four chickens were immunized with a vaccine prepared from erythrocytes of rhesus monkeys heavily infected with *Plasmodium knowlesi* which had been lyophilized and kept at about 6 C for 5 years. The vaccine was a water-in-oil emulsion of these cells with killed tubercle bacilli and was injected into 8 sites in the pectoral muscles. Each bird received about 20×10^9 parasites. These birds were challenged 6 weeks later by intravenous injection of 2.1×10^9 *P. gallinaceum*. No immunity was observed.

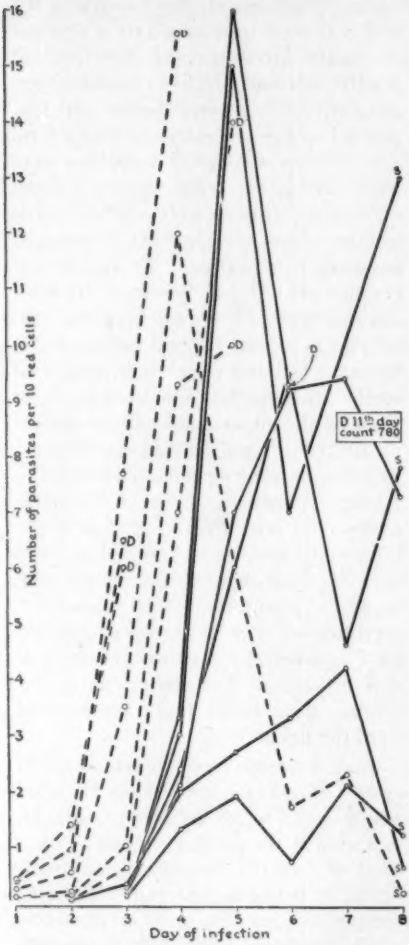

FIG. 3.—Parasite counts of *P. gallinaceum* in chickens weighing about 0.5 to 1.1 kg (1) immunized with normal chicken red cells and (2) untreated controls. Note the heterologous immunity conferred on group 1 as indicated by a slight delay in the acute rise of the infection, decreased mortality and in some birds, lower maximum parasite counts.

DISCUSSION

Immunity to blood-induced malaria in chickens immunized with normal erythrocytes of other chickens may have been caused by immune isoantibodies to chicken erythrocytes, cells which are known to vary in antigenic composition.[6] A similar immunity appears to occur in blood-induced therapeutic malaria in man, where infections induced with parasitized incompatible blood are said to have longer latent periods than those induced with compatible blood.[7] The lack of immunity in chickens inoculated with parasitized chicken erythrocytes suggests that the parasite destroys or liberates the isoantigens of the cell which it inhabits. This explanation agrees with the finding of Zuckerman[8] that opsonins for chicken erythrocytes could be absorbed out of

6. Landsteiner, K. 1947, The Specificity of Serological Reactions, Cambridge, Harvard University Press, pp. 87–89.
7. Polayes, S. G. and Derby, I. M. 1934, J. Am. Med. Assoc. **102**: 1126–1128.
8. Zuckerman, A. 1945, J. Infect. Dis. **77**: 28–59.

chicken serums more readily by normal chicken erythrocytes than by parasitized erythrocytes.

Since chickens were immunized against blood-induced malaria by inoculation with normal chicken erythrocytes, experiments in which animals are immunized against blood-induced malaria by inoculation with parasitized erythrocytes do not demonstrate a truly antiparasitic immunity unless special controls are carried out.

The experiment with ducks, in which control birds received vaccines of human or appropriately chosen duck erythrocytes, seems to show that birds immunized with parasites developed a true antiparasitic immunity and not only an isoimmunity to duck erythrocytes. This finding confirms several previous reports of immunization of birds against malaria by inoculations of killed trophozoites.[1,9–11]

In the present study, the few successful efforts to immunize were made with large birds subjected to protracted immunization with fresh vaccines.[12]

9. Gingrich, W. D. 1941, J. Infect. Dis. **68**: 46–52.
10. Redmond, W. B. 1938, J. Parasitol. **25**: (supp.) 28.
11. Jacobs, H. R. 1943, Am. J. Trop. Med. **23**: 597–606.

SUMMARY

1. Ducks (weighing initially 1.7 to 2.3 kg) immunized against *Plasmodium lophurae* by inoculations of formaldehyde-killed parasitized erythrocytes in water-in-oil emulsion with killed tubercle baccili, as previously done by Thomson et al,[1] showed increased resistance to blood-induced infection with *P. lophurae*.

2. Chickens (weighing initially 0.2 to 0.3 kg) immunized by inoculations of normal chicken erythrocytes in a similar emulsion showed increased resistance to blood-induced infection with *Plasmodium gallinaceum*.

3. Chickens (weighing initially 2.5 kg) immunized with *P. lophurae* in a similar emulsion and twice infected with *P. lophurae* showed a greatly increased resistance to blood-induced infection with *P. gallinaceum*, as compared to normal unimmunized chickens or chickens infected twice with *P. lophurae*.

12. Wolfe, H. R. and Dilks, E. 1948, J. Immunol. **58**: 245–250.

PASSIVE IMMUNIZATION OF BIRDS AGAINST MALARIA

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Following the observation of Coggeshall and Kumm (1937) that monkeys could be immunized passively against malaria, several authors reported that birds also could be immunized passively against trophozoite-induced infections with *Plasmodium circumflexum* (Manwell and Goldstein, 1940), *Plasmodium lophurae* (W. H. and L. G. Taliaferro, 1940, and Becker et al, 1949), and *Plasmodium gallinaceum* (Vezzoso, 1941 and Zain, 1944). In the present experiments passive immunity to trophozoite-induced infection with *P. lophurae* was demonstrated in ducklings and the specificity of this immunity was investigated.

MATERIALS AND METHODS

The same strains of *P. lophurae* and *P. gallinaceum* were used as in a previous study (Coffin, 1951). Hosts were white Peking ducks and chickens of mixed breed. Infection was induced by the intravenous injection of parasitized erythrocytes. Blood parasite counts were made by the methods described previously (Coffin, 1951).

Normal serums were obtained from large uninfected white Peking ducks. Immune serums throughout this paper refer to serums obtained from large ducks infected several times intravenously with *P. lophurae*. About 1×10^9 parasites were injected initially and 0.5×10^9 to 15×10^9 parasites were reinjected one or more times thereafter, at intervals varying from one week to several months. Parasites were found after the initial injection for about 10 days and reached approximate peaks of 1500 to 15,000 parasites per 10,000 erythrocytes but were usually not found after the reinjections. The procedure of obtaining the serum from artificially immunized ducks with killed *P. lophurae* and adjuvants is described in my preceding paper (Coffin, 1951).

Blood was drawn aseptically about 10 days after the last injection of parasites and was allowed to clot an hour or more at room temperature, longer if needed. The clot, after separation from the sides of the tube by a sterile wooden stick, was left at least 12 hours in a refrigerator. The serum was poured off and, after centrifuging, stored without preservative. Clots contracted poorly, yielding only small amounts of serum. Usually serums from several ducks were combined.

EXPERIMENTAL RESULTS

1. Ducklings were immunized passively against *P. lophurae* with immune serum in 4 experiments. Small ducklings were infected with *P. lophurae* and treated with serum from large ducks multiply infected 3 to 5 times with red cells parasitized with *P. lophurae*. Normal or no serum was used as a control. The number of times the donor ducks were injected with parasitized red cells and the schedule of injections into the test ducks are given in table 1 along with how many parasitized red cells the test ducks received.

Some degree of immunity was transferred with the immune serum, since the test ducks had less intense parasitemias than those receiving normal or no serum (table 1). On the other hand, large amounts of immune serum given daily for 8 days seemed no more effective than one injection administered at the time of infection (cf. ducklings receiving immune serum in experiments 1 and 2 with those in experiments 3 and 4).

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2. The in vitro effect of serum from large ducks multiply injected with parasitized red cells was tested on parasitized (*P. lophurae*) erythrocytes in 5 experiments. The in vitro contact of immune serum with parasitized red cells for 10 to 21 minutes was studied in ducklings. Normal serum was used as a control.

Blood for experiment 5 was drawn from a duck infected with *P. lophurae*, was diluted 1:20, and 1 ml diluted blood was added to 3 ml immune duck serum and 1 ml diluted blood to 3 ml normal duck serum. Both tubes were shaken gently for about 15 minutes, and a portion of the mixture from each was injected immediately into ducklings. A similar technique was used in experiments 6, 7, 8 and 9 except that, in experiment 9, the parasitized cells were separated from the serum by centrifugation, resuspended in saline and then injected into ducklings. The parasitemias in the subinoculated ducklings are shown in table 2.

The immune serums used in experiments 5, 6, 7 and 9 and 4 of the 6 serums tested in experiment 8 curtailed the parasitemia as indicated by a delay in the appearance of parasites and lower peaks of the infection. The artificially immune serum given in experiment 6 was ineffective. The effect of the immune serum was not apparently lessened by removing the serum from the parasitized erythrocytes before injection (exp. 9).

3. The effect of serum from large ducks multiply infected with parasitized red cells was tested in vivo on parasitized (*P. lophurae*) erythrocytes after ineffective in vitro contact with serums from artificially immunized ducks. In experiments 10 and 11, a combination of in vitro contact and the curative action of serum was studied.

For the in vitro contact 6 serums were obtained from large ducks successfully immunized (Coffin, 1951) by two intramuscular injections of parasitized erythrocytes in adjuvants. Four control serums were obtained from normal ducks. In experiments 10 and 11, as in experiment 5, 0.5 ml of each serum and 12×10^6 *P. lophurae*, after 6 to 9 minutes in vitro contact, were injected into 10 ducklings (weighing about 70 g in experiment 10 and about 45 g in experiment 11). No evidence of immunity could be seen in the parasite counts for the two following days.

TABLE 1.—*Passive immunization of ducklings against* P. lophurae *as shown by parasite counts, with immune serum from ducks multiply infected with* P. lophurae. *Normal or no serum was used as a control.*

Experiment	Source of immune serum			Parasites i.v.	Weight (g) of ducklings	Daily parasite counts per 1000 red cells								
	Number of ducks	Times infected	Method used			1	2	3	4	5	6	7	8	9
1	4	4	10 minutes before infection	19×10^6	43	1	5	23	88	190	672	890		90
					39	1	3	17	46	128	380	590		36
	5		1 ml i.v. 4 ml i.m.		43	6	8	54	290	410	1000	1080		34
					41	5	19	47	200	430	1310	960		80
2	4	4	As in experiment 1	19×10^6	48	0	<1	2	22	21	95	270		690
					49	4	6	39	200	440	980	920		42
3	3	7	Daily for 8 days 5–10 ml i.p. and i.m.	76×10^6	160	0	<1	3	9	24	38	29	25	9
	No serum				205	<1	1	4	20	47	104	46	7	<1
4	1	4	Daily for 9 days 6 ml i.p. and i.m.	9×10^6	74	0	2	4	27	88	170	280	700	580
	4				78	<1	3	11	46	270	540	910	1040	620
	No serum				79	<1	4	10	36	160	280	880	800	320

i.v. =intravenous, i.m. =intramuscular, i.p. =intraperitoneal.

TABLE 2.—*In vitro protective action of serum from large ducks multiply infected with parasitized* P. lophurae *red cells on* P. lophurae. *Normal duck serum or no serum was used as a control.*

Experiment	Source of serum for in vitro contacts			Parasites i.v.	Weight (g) of ducklings	Daily parasite counts per 1000 red cells								
	Number of ducks	Times infected	Ml injected			1	2	3	4	5	6	7	8	9
5	5	11	2†	4×10^6	160	0	0	1	1	2	25	96	140	90
					135	0	0	<1	3	5	25	52	100	45
					127	0	0	<1	1	3	30	45	285	390
	5				110	1	1	8	19	64	380	490	800	510
6	1	2		50×10^6	57	6	8	40	290	490	350	140		
	5	*	1		54	15	65	210	730	980	910	190		
	1				54	17	31	150	440	860	1020	290		
7	1	6	0.5	80×10^6	45	<1	<1	1	16	30				
					45	0	0	1	10	14				
					42	<1	<1	3	21	65				
					42	0	0	<1	2	6				
	1				43	5	6	56	230	440				
					45	4	18	68	280	750				
					44	5	5	40	270	530				
					40	5	12	120	280	990				
8	4	4	1†	12×10^6	85	0	<1	1	2	10	60	250		
	3	5			130	<1	<1	4	20	50	200	59		
	3	7			93	0	<1	1	5	8	29	4		
	4	4			85	0	<1	<1	<1	1	8	11		
	6	4			105	4	9	30	85	170	510	350		
	4	2			140	1	12	74	140	360	840	210		
	No serum				123	8	9	85	130	390	170	20		
9	5	11	1	57×10^6‡	5	2	4	11		90	430			
	5				5	56	150	540	1070	1100	900			

* Artificially immunized by 4 injections of formalin-killed *P. lophurae* with adjuvants; no active immunity shown.
† Per 100 g duckling.
‡ Serum removed from parasites before the parasites were injected.

Fifty-four hours later, 5 of the ducklings were given proven immune serum from repeatedly infected ducks (1 ml intravenously and 4 ml intramuscularly) and 5 were given like amounts of normal serum. Immune serums did not alter the course of infection, but one of the normal serums, which was administered to only one duckling, apparently curtailed the parasitemia. This duckling had appeared normal, but on the day after the injection of normal serum appeared anemic, and most of the erythrocytes were young forms. In 4 days parasitemia increased less than fivefold, while in the other birds in experiment 11, it increased more than fortyfold.

4. The in vitro effect of immune duck serum was studied on the infectivity of parasitized (*P. gallinaceum*) chicken erythrocytes in experiment 12. This experiment was similar to experiment 5 except that pooled serum from ducks immunized with *P. lophurae* was tested for its effect on infection with *P. gallinaceum* in chicks.

To 6 ml immune serum was added chicken blood infected with *P. gallinaceum*. Normal duck serum constituted a control. The two serum-parasite mixtures were shaken gently for 10 minutes and centrifuged. The parasitized cells were resuspended in isotonic saline and injected intravenously into chicks. Each chick received 130×10^6 parasites. As shown in table 3, the immune serum markedly reduced the parasitemia during the first few days. This finding suggested that the majority of parasites had been rendered noninfectious. Later, severe infection developed, and all eight chicks died.

This experiment was repeated with almost identical technique. Results were

TABLE 3.—*In vitro protective action of serum from large ducks multiply infected with parasitized* P. lophurae *red cells on* P. gallinaceum. *Normal duck serum was used as a control.*

Experiment	Source of serum for in vitro contacts			Parasites i.v.	Weight (g) of chicks	Daily parasite count per 1000 red cells				
	Number of ducks	Times infected	Ml injected			1	2	3	4	5
12	5	3	6	130×10^{6}*	56	0	<1	4	75	1120
					60	<1	1	8	96	900
					53	0	1	9	31	430
					53	0	<1	3	42	580
	4		6		53	5	15	210	1280	1440
					55	7	42	190	1790	1430
					60	1	6	30	400	2450
					57	<1	9	20	224	2110

* Parasites injected freed of serum.

similar except that the parasitemia in the subinoculated birds was not as effectively suppressed.

5. The effect of lysed normal erythrocytes on immune serum was ascertained in experiments 13 through 15. Immune serum was exposed to lysed normal duck red cells to observe whether the protective activity could be absorbed.

For experiment 13 about 5 ml of fresh citrated normal duck blood was centrifuged. The cells were then washed by resuspending and recentrifuging in normal saline and were lysed by resuspending in distilled water. The lysed residues were washed several times in saline until they became white and were then packed by recentrifuging. One and one-half ml of packed lysed erythrocytes was suspended in 3 ml of immune duck serum (the immune serum used in experiments 1 and 2). Saline plus immune serum acted as a control mixture. Both mixtures were agitated gently for 45 minutes at room temperature and then centrifuged. The supernatants were drawn off for use in the following in vitro protection tests (by the method used in experiment 5): One-half ml of freshly drawn and diluted blood from a duck infected with *P. lophurae* was suspended in 2 ml of cell-treated serum, and a similar amount in 2 ml of saline-treated immune serum. Both mixtures were shaken gently. Between 9 and 15 minutes later, two groups of 4 ducklings were infected intravenously with 0.4 ml of one of the mixtures.

Parasite counts shown in table 4 indicate that the protective activity of immune serum was largely removed by contact with lysed normal duck erythrocytes. In experiment 14, to observe whether absorption of the immune property was a specific effect of duck cells, residues of lysed human erythrocytes (A, Rh-positive) were prepared in the same manner and suspended

TABLE 4.—*Absorption by lysed normal duck red cells of the protective action of serum from large ducks multiply infected with parasitized* P. lophurae *red cells. Unabsorbed immune duck serum was used as a control.*

Experiment	Source of serum for in vitro contacts			Parasites i.v.	Weight (g) of ducklings	Daily parasite counts per 1000 red cells				
	Number of ducks	Times injected	Ml injected			1	2	3	4	5
13	4	4	0.4 (absorbed)	10×10^{6}	70	0	<1	<1	4	13
					68	<1	<1	7	3	18
					67	<1	<1	2	3	13
					61	0	<1	1	6	46
	4	4	0.4 (unabsorbed)	10×10^{6}	69	0	0	0	<1	<1
					65	0	0	<1	<1	4
					63	0	0	<1	1	2
					69	0	0	<1	1	2

in a fresh sample of the same immune serum as in experiment 13. The cell-treated and saline-diluted serums were used in experiment 14 in exactly the same manner as in experiment 13. The protective effect of the immune serum was not absorbed by lysed human erythrocytes.

In experiment 15 erythrocytes from an immune duck were tested for their ability to absorb the protective substance from immune serum from the same duck. After 1.2 ml packed saline-washed residues of lysed erythrocytes were suspended in 3.5 ml serum, were agitated gently for 45 minutes at room temperature and centrifuged, the supernatant was injected as in the two preceding experiments. Saline plus immune serum were treated similarly. The red cells from one duck appeared to absorb to some extent the protective property of its immune serum. (This was repeated with similar results.) Since the protective property was very slight the experiment was repeated with a stronger serum. Red cells from another immune duck, however, failed to absorb the protective property of its immune serum. In other words, the protective property was still present when tested in vivo.

6. Normal duck erythrocytes were found to be agglutinated by serum from ducks immune to *P. lophurae*. Some of the foregoing experiments suggested that serums of ducks immune to *P. lophurae* contain antibodies to normal duck erythrocytes. Agglutination could be detected in some serums by suspending 0.05 ml packed washed normal duck erythrocytes in 1.0 ml undiluted serum and allowing the suspension to stand for 1 hour at 37 C. In strong agglutinating serum, the cells settled in large clumps which could be broken to form a homogeneous suspension only by brisk shaking. With nonagglutinating serums, the sedimented cells were not clumped and could be easily resuspended by gentle tilting. Six proven immune serums and 8 normal serums were tested in this way against fresh red blood cells of one duck. All of the immune serums produced marked agglutination, one normal serum produced slight agglutination and the others no agglutination. One of the immune serums was tested against cells from 15 uninfected ducks and produced strong agglutination in each. The normal serum when tested again produced moderate agglutination.

Although immune duck serums were not tested with chicken erythrocytes, serums from rabbits immunized with normal duck or chicken erythrocytes agglutinated normal erythrocytes of both ducks and chickens.

DISCUSSION

The present results extend to the duck the findings of earlier authors (Manwell and Goldstein, 1940; W. H. and L. G. Taliaferro, 1940; Vezzoso, 1941 and Zain, 1944) on the passive transfer of immunity against malaria in birds and the findings of W. H. and L. G. Taliaferro (1940) on the action of immune chicken serum on parasitized erythrocytes in vitro. They suggest that much of the effect of immune serum, especially hyperimmune serum from birds which have received repeated transfusions of parasitized erythrocytes, is produced by antibodies against the enveloping erythrocyte rather than antibodies against the parasite.

In support of the presence of anti-erythrocytic antibodies, each of 6 immune serums known to have protective activity could agglutinate normal duck erythrocytes, and the protective activity of one of them could be absorbed out by lysed normal duck erythrocytes. In this respect, the results agree with the in vitro opsonic studies of Zucker-

man (1945) who found opsonins in hyperimmune serums from chickens superinfected with erythrocytes parasitized with *P. lophurae* and *P. gallinaceum* but not in serums of chickens recovering from initial infections. The opsonins acted on either normal or parasitized erythrocytes and could be absorbed out of the serums more readily by normal than by parasitized erythrocytes. Becker et al (1949) also produced passive immunity with an apparently antierythrocytic serum.

Apparently antiparasitic antibodies are also present. Dr. L. A. Stauber (personal communication) has found that our immune serums agglutinate cell-free *P. lophurae*. One serum was absorbed with normal duck erythrocytes and retained its agglutinating activity undiminished. Since the in vitro protective activity of some of our serums could be absorbed out by normal duck erythrocytes, it seems unlikely that much of the protective action of these serums was produced by antiparasitic antibodies. Moreover, our immune serums were administered in such a way that there was opportunity for the antierythrocytic antibodies to act on donor erythrocytes before the contained parasites had escaped and begun to multiply in the host. None produced detectable passive immunity after parasite multiplication had begun. However, in some of the experiments of Coggeshall and Kumm (1937) and Mosna (1938), passive immunity was produced in monkeys after the parasites had begun to multiply, and Vezzoso (1941) demonstrated passive immunity with serum which was obtained from sporozoite-infected chickens and which may have contained no antibodies to erythrocytes. Thus, not all passive immunity in malarias is caused by antibodies to donor erythrocytes. Trager and McGhee (1950) obtained passive immunity with plasma from normal adult ducks and chickens and reviewed evidence that the protective substance may be a lipoprotein whose concentration is augmented during activity of the ovary or after recent malarial infection. Since passive immunity may be produced by such a substance or by antibodies to erythrocytes, the author believes that circulating protective antibodies specific for avian plasmodia have not been demonstrated.

The antierythrocytic antibodies in the present studies may have been produced in response to repeated injections of duck blood. Isoagglutinating activity was also shown in the serum of one normal duck. However, protective serum was obtained from birds which had been infected with *P. lophurae* and not from the birds immunized artificially with parasitized erythrocytes. In agreement with the suggestion that the malarial infection itself caused the production of antierythrocytic isoantibodies are the findings of Heidelberger and Mayer (1944) and Oliver-González (1944) who found such isoantibodies in serums of persons who had malaria and blackwater fever, respectively.

If autoantibodies able to react with the donor's own cells were present in our serums, as the slight autoabsorption in the serum of one duck in experiment 15 suggests, they may have contributed to the active immunity of the donor against malaria by assisting in the destruction of parasitized and normal erythrocytes during the acute parasitemia. A marked destruction of erythrocytes occurs in avian malarias and appears to assist in reducing the parasitemia. Thus, if fresh erythrocytes are injected, parasitemia is maintained (Rigdon and Varnadoe, 1945 and Becker and Marousek, 1949) and, conversely, if hemolytic substances are administered

during parasitemia to human subjects (Lorant, 1946) or monkeys (Cruz, 1949), the parasitemia is curtailed. Instances of phagocytosis and of intravascular agglutination of normal erythrocytes during acute parasitemia in the malarias have been reported (Taliaferro and Cannon, 1936; Malamos, 1937 and Lack, 1942) and suggest that autoantibodies to erythrocytes are present.

The single observation, recorded in experiment 11, of increased resistance in a duckling after it received one dose of normal duck serum, was unexpected. In this instance the serum had no opportunity to act on donor erythrocytes. (This bird had received earlier an apparently ineffectual dose of serum from an artificially immunized duck.) The replacement of mature erythrocytes by young forms, just after the injection of normal serum, suggested that the normal serum was hemolytic and produced its protective effect by destroying the host's erythrocytes, both parasitized and unparasitized, more quickly than the parasite could multiply. However, a hormone, nutrient or other substance may have been the active ingredient of this serum.

SUMMARY

1. Ducklings infected with *Plasmodium lophurae* by the intravenous injection of parasitized blood were immunized passively by immune serum given at the time of infection. The immune serum was obtained from ducks which had been infected repeatedly with *P. lophurae* by injections of parasitized blood.

2. Parasitized blood from ducks infected with *P. lophurae* was rendered less infectious by contact in vitro with immune serum.

3. The in vitro protective activity of immune serum was much reduced after contact with lysed normal duck erythrocytes.

4. Immune serums agglutinated normal duck erythrocytes.

5. Parasitized blood from chickens infected with *Plasmodium gallinaceum* was rendered less infectious for chicks by contact in vitro with immune serum from ducks repeatedly infected with *P. lophurae.*

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THE DISTRIBUTION OF SPARING AND PROTECTIVE FACTORS IN PLASMA FRACTIONS OF DUCKS RECOVERED FROM LOPHURAE MALARIA

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There was described in two previous reports[1,2] an immunological reaction called the sparing phenomenon.[3] The circumstances for observing the phenomenon are as follows: One group of young chicks receives intravenously a certain amount of physiological salt solution; another group the same volume of plasma from ducks recovered from infections of *Plasmodium lophurae*. Then, one and one-half hours later both groups of chicks are injected intravenously with uniform doses of washed duck cells parasitized with *P. lophurae*. Blood smears of both series are made from time to time. If the behavior of the groups is typical, readings of the blood smeared from a few minutes to three days (more or less) after the time of the red cell injection record higher mean percents of parasitized erythrocytes in the recipients of immune plasma than in the recipients of physiological saline, because the injected plasma in some manner or other spares the injected duck cells from the full impact of the bodily defenses of the chick on the foreign cells in its circulation. The sparing process is ordinarily operative before the protective effect (passive immunity) of the injected immune plasma becomes evident.

The discovery and confirmation of the sparing phenomenon were based on the results of injecting complete plasma. Further tests were made with plasma fractions in order to learn more about the nature of the sparing factor, as well as of the protective factor,[1–4] and to explore the possibility of obtaining a plasma product with relatively less sparing factor and more protective factor. So far as can be learned, the only workers to experiment with the protective properties of fractions of plasma were Trager and McGhee,[5] who obtained a degree of passive immunity against *P. lophurae* in chicks by injecting them with "euglobulin" precipitated out of aqueous dilutions of plasma from egg-laying hens.

MATERIALS AND METHODS

The results reported in the present paper are based on comparisons of the mean percents of parasitized erythrocytes in control chicks, either injected with physiological salt solution or uninjected, and in chicks injected with comparable volumes of diluted plasma precipitates from

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immune (i.e., "latent") ducks. The ducks serving as the source of plasma were half- to fully grown, had recovered from blood-induced lophurae malaria, and their blood had been found to be microscopically free of the parasite before it was drawn. The protozoon was carried in the laboratory by passing through ducks. The procedures were in general the same as previously outlined.[1,3]

The preparation of plasma fractions requires special description. Blood was drawn from the right jugular vein of ducks from whom food, but not water, was generally withheld for 4 or 5 hours beforehand. Coagulation was prevented with either heparin or citrate, as was previously described.[1,3] The plasma of not less than 4 ducks was pooled in a beaker, and chilled to 2 to 4 C in a bath of iced water. Fractionation was performed with various concentrations of ammonium sulphate (reagent), using Hawk, Oser and Summerson[6] for reference. Special care was taken that the ammonium sulphate solution was saturated with the salt several days before the fractionation was made. The plasma was kept chilled in a beaker sitting in iced water and stirred constantly with a glass rod as the saturated salt solution was added drop by drop from a burette at room temperature. There follows an account of the preparation of 4 plasma fractions, viz., fraction A ("fibrinogen") precipitated at 20% saturation with ammonium sulphate, fraction B ("euglobulin") precipitated at 20% to 33.3% saturation, fraction C ("pseudoglobulin I"+"pseudoglobulin II") at 33.3% to 50% saturation, and fraction D ("albumin"), the supernatant over fraction C.

To 100 cc of pooled plasma was added 25 cc of the saturated salt solution. The precipitate was separated either by centrifuging in a chilled centrifuge or by collecting on Whatman no. 50 hardened filter paper. In the latter case the filtration was made in a refrigerator at about 5 C and often required many hours. The residue, in the bottom of the 50 cc centrifuge tube or scraped from the paper, was dissolved in cold physiological salt solution, made up to 100 cc and reprecipitated.

Usually a second reprecipitation was made. The final cold white residue was dissolved in about 50 cc of physiological salt solution, poured into a Visking bag, tied off, dialyzed about three hours in several changes of cold tap water, then in cold physiological salt solution (refrigerator temperature) changed frequently until no more sulphate was present as determined by the barium carbonate test. The volume was equated to the original volume of the plasma, i.e., 100 cc. This whitish opalescent solution constituted fraction A and was kept frozen in the refrigerator until needed.

The supernatant or filtrate was assumed to be 20% saturated. It was measured, and to it was added enough of the salt solution to make 33.3% saturation. The residue was separated, redissolved, reprecipitated twice, and dialyzed. In this case also the final precipitate was cold white, and when redissolved in physiological salt solution and equated to 100 cc made an opalescent solution, fraction B.

This filtrate or supernatant was now considered to be 33.3% saturated with ammonium sulphate, and it was brought to 50 to 51% saturation. The residue was separated by filtering or centrifuging and dissolved in physiological salt solution. One reprecipitation only was made. Dialyzing and dilution to 100 cc were carried out as in the case of the other fractions. The solution obtained, fraction C, was clear, i.e., not opalescent, and had a very light color of brown.

The supernatant was now considered to be 50% saturated with the salt. It should be clear and yellow. If any cloudiness remained in it after standing several hours in the refrigerator, it was filtered again in the refrigerator; but when this filtrate was still cloudy it was passed through a Seitz bacto-filter disc with the aid of suction. The clear filtrate in a Visking bag was dialyzed in cold running tap water for 4 hours. Great increase in volume of solutions in Visking bags was prevented by tying off the bag near the surface level of the fluid. So far none of the membranes thus treated have ruptured. Then the bag was immersed in distilled water in a refrigerator and dialysis proceeded with frequent changes of the water until the barium carbonate test showed the absence of sulphate from the water.

Volume was reduced to less than the original volume of plasma by subjecting the membrane to a stream of air from a fan. After adjusting the volume to 100 cc, sodium chloride concentration was brought to normality by adding the solid salt. This clear yellow solution constituted fraction D.

RESULTS

The results of the tests, i.e., means and standard deviations of percents of parasitized cells, and information relative to age of chicks when inoculated, number of parasitized duck erythrocytes injected per 100 g of chick body weight, and amount and frequency of

6. Hawk, P. B., Oser, B. L. and Summerson, W H. 1947, Practical Physiological Chemistry, ed. 12, Philadelphia, Blakiston Co.

fractions injected per 100 g of chick body weight appear in the tables unless stated in the text. The order of presentation of the results will be by fractions instead of by series of experiment.

Fraction A.—This fraction, precipitated at 20% saturation with ammonium sulphate, was tested for sparing and protective activity in 7 series. The volume injected into the veins of the test groups varied from 0.6 to 1.0 cc/100 g of chick body weight. Control groups either received the same volume of physiological salt solution or were uninjected. The solutions were injected on days 0 to 1 in series E, days 0 to 2 in series D, days 0 to 3 in series B, days 0 to 4 in series A, C, and G, and days 0 to 5 in series F. The fraction A-recipients in the 7 series numbered 45 chicks and the controls 46 chicks. There were also groups in each of the 7 series that received either whole immune plasma or fractions exhibiting sparing activity.

TABLE 1.—*Results of testing fraction A for sparing and protective activities.*

Series	Group and treatment	No. of chicks	Data:	Time after inoculation									
				1 hr. ±	1 da. ±	2 da.	3 da.	4 da.	5 da.	6 da.	7 da.	8–9 da.	10 da.
D	1 (saline control)	6	M	1.73	10.6	10.4	43.5	51.7	46.3	25.5	10.2	1.7	1.3
			SD±	0.16	4.0	4.0	13.4	5.9	8.9	12.1	12.5	2.1	1.4
	2 (fraction A)	6	M	1.88	9.0	9.7	36.3	33.9	15.6	2.9	0.9	0.1	0.1
			SD±	0.17	3.4	4.4	7.4	9.2	10.0	5.1	1.2	0.1	0.2
G	1 (saline control)	8	M		1.1	3.3	9.0	15.6	27.4		11.6	12.5	(10.5)*
			SD±		1.2	3.9	9.8	16.5	26.7		14.2	15.8	(12.3)
	2 (fraction A)	8	M		0.3	1.0	3.0	5.5	11.6		4.1	0.2	(+)*
			SD±		0.4	1.8	4.5	10.5	9.2		7.9	0.3	(+)

* 15 days.

It has been observed previously[2] that the sparing effect is generally most pronounced in chicks a day or two after the injection of plasma and parasitized erythrocytes. Inspection of the mean percents of parasitized cells for day 1 and day 2 revealed that, standard deviations considered, the counts of the 7 test groups were practically the same as, or less than the mean counts of their controls. In series G (table 1) the mean of the test group on day 1 was significantly lower than that of the control, while on day 2 it was still considerably lower, though not significantly so. It is safe to conclude that the 7 tests did not disclose the presence of appreciable amounts of sparing factor in fraction A, but did the record indicate that it contained the protective factor?

The data for day 3 showed that in 6 series the means of the fraction A-recipients were less than those of the controls, but in series C the reverse was true, although the difference was far from significant. If the chances were equal that the means of the test groups would be lower than those of the controls, the probability of obtaining 6 test means lower than the controls in 7 series would be 0.0625, according to the binomial distribution. When the difference between means on day 3 to day 7, inclusive, in each of the series was tested for significance by the Fisher's small sample method previously described,[1] none was significant except that of day 6, series E, and those of day 5, day 6, and day 7, series D (table 1). Since a considerable part of the magnitude of the standard deviations appeared to be due to irregularities in the course of the individual

infections and in the day on which the peak was attained, it appeared that it would be altogether sound to use the peaks attained in the individual birds between days 3 and 7, inclusive, as the variables.

In series A the mean of the peaks for group 1 was 59.8 ± 10.9; that for group 2, 36.4 ± 17.1. When tested by the aforementioned small sample method the difference proved significant with $P = 0.05$.

appear in the captions of table 2. The same statements applied to series O also, except that the injections were made on days 0 to 4, and there were 9 chicks in each group. Group 3 (controls) and group 4 (tests) of series N (table 2) showed no significant differences at 2 hours, 1 day, 2 days, or 3 days, but a wider disparity between the means developed subsequently. On days 4 and 5 the differences were not yet significant,

TABLE 2.—*Series N. (Age 11 days; 3.5×10^8 duck P.C.; 0.8 cc days 0 and 1).*

Group and treatment	No. of chicks	Data:	Time after inoculation								
			2 hr.	1 da.	2 da.	3 da.	4 da.	5 da.	6 da.	7 da.	8 da.
1 (plasma less fraction A)	5	M	1.20	0.69	0.51	3.1	3.9	5.2	4.1	0.7	0
		SD±	0.36	0.5	0.45	2.1	3.5	3.6	6.4	0.8	
2 (fraction C+D)	8	M	0.87	1.71	2.83	9.8	17.8	22.7	19.1	15.2	3.5
		SD±	0.55	0.33	1.00	3.2	6.0	3.5	19.9	17.7	3.6
3 (saline, control)	8	M	0.65	0.59	0.85	3.8	11.8	20.5	34.7	21.6	6.0
		SD±	0.35	0.30	0.77	3.5	9.4	16.1	25.5	9.9	2.6
4 (fraction B)	8	M	0.58	0.47	0.93	2.7	6.5	8.7	6.0	1.8	0
		SD±	0.45	0.36	0.96	2.5	6.6	7.5	5.7	3.9	

In series G the means of the peaks for group 1 and group 2 during the same interval were, respectively, 35.4 ± 20.2 and 12.9 ± 10.3, and the difference was significant with P between 0.01 and 0.02. This treatment did not yield significant differences in series B or series F. To summarize, significant or highly significant differences indicating a protective factor in fraction A were obtained in series A, D, E, and G. On days 3 and 4 the means of the fraction A-recipients were lower than those of the controls in series B and F, but the differences were not significant. Only in series C were the means of the fraction A-recipients (insignificantly) higher than those of the controls.

Fraction B.—This fraction, precipitated in the range of the saturation (ammonium sulphate) scale between 20% and 33.3%, was tested in series N (table 2) and series O. The amounts and frequency of the injections for series N

but they became highly significant on days 6, 7, and 8. Group 1 (tests) and group 3 (controls) of series O showed no significant differences at 4 hours and 1 day, but by day 2 $P = 0.02$, which proved the sparing factor was operating. There was no evidence of protective properties in the sample of fraction B used in series O. In summarizing it can be said that one sample of fraction B (series N) showed no sparing properties and strong protective properties, while another sample (series O) showed marked sparing properties but afforded no protection. There was probably very little protective antibody in the plasma employed in series 4-O because the whole plasma (group 4), fraction C (group 2), and fraction B+C (group 5) each failed to confer any degree of passive immunity.

Fraction A+B.—This, the precipitate at 0% to 33.3% saturation with ammonium sulphate, represented ap-

TABLE 3.—*Series Q. (Age, 11 days; 3.0×10⁸ duck P.C.; 1.6 cc on day 0, except G2, no injection).*

Group and treatment	No. of chicks	Data:	Time after inoculation											
			1 hr.	6 hr.	1 da.	2 da.	4 da.	5 da.	6 da.	7 da.	8 da.	10 da.	11 da.	12 da.
1 (fraction A+B)	7	M	1.22	1.01	1.48	5.24	27.9	16.7	19.9	7.5	4.4	0.32	+	0
		SD±	0.34	0.29	0.62	1.86	13.2	11.0	13.8	10.0	7.5	0.69	+	0
2 (control)	7	M	0.19	0.11	0.27	1.19	7.8	6.7	24.9	24.1	28.1	10.78	1.85	0.23
		SD±	0.28	0.10	0.30	1.01	7.4	4.5	13.9	9.9	16.1	10.09	1.96	0.24
3 (fraction C+D)	7	M	1.36	1.74	2.61	8.70	45.7	34.7	39.9	15.3	13.3	1.65	+	+
		SD±	0.42	0.62	1.46	3.10	9.6	8.1	15.4	10.4	12.7	2.27	+	+

proximately the sum of fraction A and fraction B. It was tested for sparing and protective activity in series L, P, Q, R and S representing totals of 42 chicks in the test groups and 53 in the control group. The average injection was about 0.8 cc and was given an average of about three times beginning with day 0.

Three series, L, P and Q (table 3), each contained a group of fraction A+B-recipients which exhibited the sparing phenomenon, significantly, early in the infection, while in the two series R (table 4) and S there was produced no evidence for the presence of the sparing factor in the fraction. All 5 series exhibited passive immunity conferred by the injected plasma, and the differences are either significant or highly significant at some time after the 5th day, except in one series, L. In the last instance the control chicks, although the mean count of the group was about 12 times that of the test group on the 7th day, were such a variable lot that the difference was not statistically significant.

Fraction C.—This fraction of immune duck plasma, separated at 33.3% to 50% saturation with ammonium sulphate, was tested in 5 series, K, L, O, P and R (table 4). In two other series, N (table 2) and Q (table 3), fraction C was not separated from fraction D; but since, as will be proved later, fraction D is relatively inert, it seemed proper to include these also as tests of the activity of fraction C. There were totals of 65 test chicks and 58 control chicks in the 7 series. The information given in the captions of tables 2 and 4 is indicative of the treatment in the other series, except Q.

Seventeen chicks composing the tests (group 3) of series K each received 1 cc of fraction C on day 0 only, and the 10 control chicks (group 2) the same amount of physiological saline. The number of parasitized duck erythrocytes injected was $3\times10.^{8}$ Means for

TABLE 4.—*Series R. (Age, 14 days; 2.0×10⁸ P.C.; 1 cc on days 0, 1, 2, 3).*

Group and treatment	No. of chicks	Data:	Time after inoculation										
			45 min.	1 da.	2 da.	3 da.	4 da.	5 da.	6 da.	7 da.	9 da.	11 da.	13 da.
1 (control)	11	M	1.7	2.5	4.7	18.2	22.9	24.6	27.1	23.5	10.7	9.9	12.9
		SD±	0.6	1.8	3.5	12.1	15.0	17.1	20.7	22.3	15.6	11.6	18.3
2 (fraction A+B)	11	M	1.7	3.2	5.9	19.1	19.8	15.4	12.4	10.7*	3.7*	2.2*	0.2*
		SD±	0.5	2.3	3.9	10.9	10.2	13.2	17.6	20.5	8.6	4.7	0.6
3 (fraction C)	11	M	1.9	3.9	7.6	28.8	30.7	37.1	22.7	17.9	6.7	1.0	0.1
		SD±	0.2	2.2	4.3	17.3	15.3	15.6	13.8	20.9	14.7	1.4	0.3

* 10 survivors.

test and control groups on day 1 were 2.62 ± 1.65 and 1.24 ± 1.00, respectively, and on day 2, 2.39 ± 1.32 and 1.29 ± 0.94. The differences are significant. Thereafter through 13 days the group 3 infections pursued a slightly higher course than group 2 without significant difference.

Inspection of the data of 45 minutes to 4 days for fraction C- and fraction C+D-recipients and for the controls in the other 6 series, viz., L, N (table 2), O, P, R (table 4), and Q (table 3), revealed that in these tests also the test chicks gave notable and authentic demonstrations of the sparing phenomenon so convincing as to require no analytical proof.

The real issue is whether any of the groups of fraction C-recipients exhibited passive immunity after the 4th or 5th day. It is stated above that the test group in series K did not. Neither did the tests in series L, O, Q (table 3), or R (table 4). In series P, however, there were wide differences between test and control means, but the standard deviations were so high that the differences were not really significant until after 10 days. The means of the peaks for this series from days 6 to 10 were 22.2 ± 19.7 for the test and 6.3 ± 5.2 for the control groups, but the difference is only nearly significant. There were also wide differences between the means and tests in series N on days 6, 7, and 8, but here again none is actually statistically significant on account of the variability in the groups. Testing the means of the peaks of the infections did not help much toward establishing significance.

Thus, the sparing phenomenon was exhibited in fraction C- and fraction C+D-recipients in all of 7 series. Protection was definitely not conferred on 5 of the test groups, but may have been conferred on two, although the differences are not statistically significant.

Fraction D.—This fraction, the supernatant over the precipitate at 50% to 51% saturation with ammonium sulphate, was administered to the test groups of 8 series comprising in all 60 chicks. The volume injected into the test chicks varied from 0.5 to 1.0 cc/100 g of chick body weight. The injection was made on day 0 only in series K, days 0 and 1 in series H and 5-E, days 0 to 2 in series 4-C, and days 0 to 4 in series E, G, I, and J. There was a total of 46 chicks in the 8 control groups.

An inspection of the means and standard deviations of test and control groups of the 8 series revealed that, taking standard deviations into consideration, in only series H was there any likelihood that sparing action was exhibited on day 1 or day 2. In this instance $P = 0.1$ to 0.2 on day 1 and 0.01 to 0.02 on day 2. Thus this difference was significant, particularly on day 2. A similar inspection of the data made for protective action of fraction D, which if occurring should commence to develop on days 3 to 5 and continue for some time afterwards, brought out no significant differences between test and control groups. Thus it is evident that in an altogether adequate series of tests little or no sparing factor or protective factor is ordinarily present in fraction D.

Series M was designed to test something that was suspected, i.e., that there is a considerable amount of sparing factor in the range of the saturation scale between 45% and 51%, inclusive. Group 1, 17 chicks, was injected on days 0 and 1 with 1 cc of the supernatant over the precipitate at 44% saturation with ammonium sulphate. Group 2, 8 chicks injected with saline, served as one control group, while group 3, 9 chicks injected with fraction D, served as another. The mean counts of the three groups on day 1 were, respectively, 1.17 ± 0.90, 0.14 ± 0.31, and 0.30 ± 0.28.

The test group is significantly different from each of the controls on this day and also on days 3 and 4. On the latter day the counts were, respectively, 15.0 $\pm$11.3, 2.3$\pm$4.4, and 3.4$\pm$3.7.

Other fractions.—Since it had been established that fraction C contained notable amounts of sparing factor and that certain samples of fraction B and fraction A+B were lacking in effective amounts of sparing factor, it was conjectured on a purely hypothetical basis that its presence in the B-component might be due to its precipitation in the upper scale of the 0% to 33.3% saturation range. Two series were studied in accord with this hypothesis, in one of which (series T) the test chicks received "fraction E" precipitated in the 0% to 30% saturation range, in the other of which (series G) the tests received "fraction G" precipitated in the 0% to 27.5% range. Thus both fractions included all of fraction A and a considerable part of fraction B also.

Series T was set up as follows: group 1, 14 chicks, recipients of fraction F, precipitated between 31% and 46% saturation with ammonium sulphate; group 2, 20 chicks, recipients of fraction E; group 3 or control group, 10 chicks, recipients of physiological salt solution.

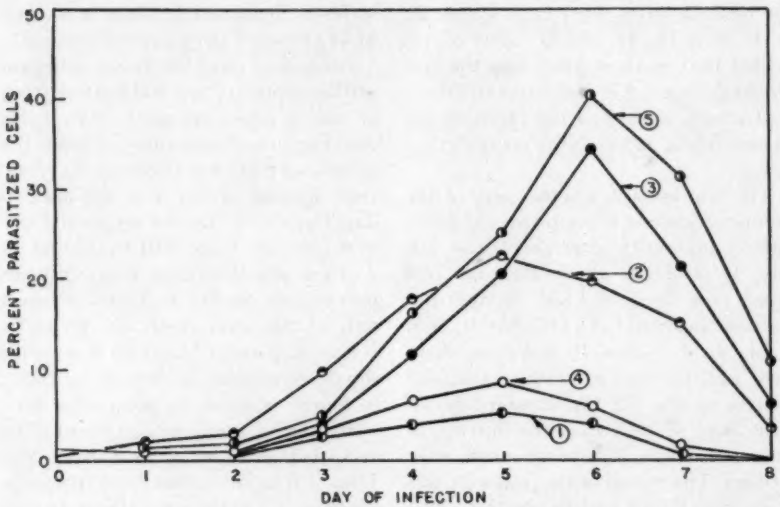

FIG. 1.—Course of infection in 5 groups of chicks (series N) injected with *Plasmodium lophurae* in duck cells and receiving 5 different treatments: 1. Recipients of immune duck plasma with "fibrinogen" removed; 2. recipients of fraction C+D; 3. control group, recipients of physiological salt solution; 4. recipients of fraction B; 5. recipients of whole plasma from a "recovered" duck taken in relapse.

The solutions were administered in 1 cc amounts on day 0 and 2 cc amounts on day 2. The experiment was disappointing for two reasons: the chicks in general happened to be an unusually resistant lot, for most of the control infections were unusually light; and the natural resistance of individual chicks was extremely variable, as indicated by the high standard deviations which on almost every day were two to three times the mean. At 3 days the means for

groups 1, 2, and 3 were, respectively, 8.2 ± 8.2, 3.2 ± 6.8, and 0.2 ± 0.7. Group 1 is significantly different from group 3, while group 2 is not. At 5 days they were, respectively, 23.4 ± 19.6, 9.4 ± 13.1 and 3.1 ± 8.6. After 5 days the data suggest continued differences between the groups, but they are not even nearly significant. Regardless of statistical analysis it is believed that the data for days 3 and 5 together do indicate that the sparing phenomenon was demonstrated in groups 1 and 2, that there was more sparing factor present in fraction F (group 1) than in fraction E (group 2), and that both fractions were deficient in protective factor, although group 1, as would be expected, showed even less evidence of protection than group 2.

Series G, for testing fraction G precipitated between 0% and 27.5% saturation, was set up as follows: group 1, 8 chicks, saline-recipients; group 3, 8 chicks, recipients of fraction G. Solutions were administered in 0.8 cc amounts on days 0 to 4, inclusive.

The spread between the means of groups 1 and 3 was very wide on all days and indicated strong protective properties of the plasma, but here again the standard deviations were large enough to defeat obtaining a significant probability on any day. The means of the peaks through 9 days for group 1 and group 3, respectively, were 34.3 ± 21.6 and 11.7 ± 10.0, with $P = 0.03$, indicating a significant difference.

Series N is especially interesting because the plasma from which fraction A had been removed showed potent protective properties but no sparing action (table 2, group 1; fig. 1, curve 1). Fraction A from this plasma employed in series A (see above) produced no sparing action but showed protective properties. Fraction B, as previously stated, demonstrated no sparing action and proved to be potent in protective properties (table 2, group 4; fig. 1, curve 4). Fraction C+D (table 2, group 2) demonstrated sparing activity from one day to 4 days. Graph 2 (fig. 1) for this fraction does not reach the peak attained by graph 3 (the control group) on the 5th day, although it has a better start. In fact, its ascent relative to curve 3 slows down beginning at 4 days, and the decline begins at 5 days while graph 3 is still rapidly ascending. Although, as stated above, the differences between the means of the fraction C+D-recipients and control group on days 6 and 7 are not significantly different, the graph indicates this fraction contained some antibody. Curve 5 of figure 1 represents the course of the infection in a group of chicks receiving the plasma of an adult duck taken in relapse. It is quite obvious that the relapse plasma did not contain enough antibody to confer passive immunity.

Table 3 and the graph (fig. 1) tell the story of a plasma sample that contained enough sparing factor to reproduce the sparing phenomenon when the fraction containing it (C+D) was segregated but which in its entirety was so potent in protective antibody that sparing activity was masked.

DISCUSSION

The results obtained in testing the fractions for sparing and protective properties, both observed previously in chicks injected with whole duck plasma, exceed expectations, for they prove not only that the factors producing sparing and protective phenomena are proteins, but also that they predominate in different components of the plasma. Fraction A, precipitated by ammonium sulphate in the 0% to 20% saturation range, usually contains demonstrable amounts of the protective factor but no sparing factor. Fraction B ("euglobulin"), coming down in the 21% to

33.3% saturation range, contains the bulk of the antibody if it is present in the plasma in effective amount, and may or may not contain the sparing factor. It is reasonable, of course, that fraction B should fail to exhibit protective activity when the whole plasma and other plasma fractions also fail in this respect, as in series O.

Fraction C ("pseudoglobulin I" +"pseudoglobulin II"), precipitated in the 33.4% to 50% saturation range, was strong in sparing activity in the 7 groups tested, and is thus proved to contain the bulk of the sparing substance. There were indications of a small amount of protective activity in several of these series, but statistical significance could not be established. The course of graph 3 of series N (table 1) would indicate that in this instance fraction C+D contained a minor part of the protective antibody. The orange-yellow supernatant over fraction C, called fraction D, contains most of the albumin and the other nondialyzable components of plasma not precipitated by full 50% saturation with ammonium sulphate. This component was found to be relatively inactive as regards sparing and protection in 7 of 8 groups tested, although in one test (series H) significant sparing activity was noted. The results obtained in series M indicate that there may be a heavy concentration of sparing factor in the material brought down in the 45% to 51% saturation range.

Although the incentive for these tests was purely academic, it was clearly recognized that efforts should be made in the direction of producing plasma fractions possessing strong protective properties not combined with sparing activity.

Since there were indications that the bulk of protective antibody was in fraction A and fraction B, 5 tests (series L, P, Q, R, S) were made of the combination of the two. In all 5 series there were rather striking exhibitions of passively conferred immunity. In three of the tests sparing effect was apparent early in the course of the infections, but in two series which, incidentally, included moderately large numbers of chicks (series R, table 4; series S) it was not to be noted. Thus it was proved that by segregation a plasma product can be obtained that either lacks appreciable amounts of sparing substance or possesses such potent concentrations of protective antibody that the sparing factor is masked.

Since three of the 5 samples of fraction A+B tested did exhibit demonstrable sparing activity, two efforts were made to eliminate it by limiting the upper range of saturation with the salt. Fraction E of series T was precipitated in the 0% to 30% saturation range, fraction F in the 31% to 46% range, both from the same sample of pooled plasma. Fraction F conferred significant sparing activity while fraction E did not. It could not be determined whether the lower counts for the fraction E-recipients relative to the fraction F-recipients were due to less sparing substance or more antibody. It is, however, obviously impossible to segregate fractions potent in protective antibody when the whole plasma lacks a considerable concentration of it.

Fraction G of series G, precipitated in the 0% to 27.5% saturation range, did not exhibit sparing activity but conferred significant protection. This series gave some indication of improved segregation of the protective factor, but obviously there is still required further testing of the basic tenet, viz., that relatively more protective factor and relatively less sparing factor are obtainable by reducing the upper limit of the saturation range.

Would potent antibody segregations prove superior to whole immune duck plasma in the treatment of lophurae

malaria in ducks inoculated with parasitized duck erythrocytes or sporozoites? Circumstances forced us to discontinue our bird inoculation experiments before that problem could be investigated. It could be argued that segregating the protective antibody from the sparing factor would not be advantageous, because the blood of the infected duck would normally contain so much of the latter that the injection of a little more or less would make no difference. But if, on the other hand, it should eventually be proved that sparing activity and protective activity are functionally balanced in the duck, then it might be possible to create an imbalance unfavorable to the parasitized cell by injecting antibody without sparing factor. The problem is exceedingly complex, and could be settled only by actual testing in a large series of experiments.

CONCLUSIONS

1. The bulk of the sparing factor in immune duck plasma is concentrated in the protein fraction precipitated by ammonium sulphate in the 33.4% to 50% saturation range.

2. The bulk of the factor that confers a degree of passive immunity is concentrated in the protein fractions precipitated by ammonium sulphate in the 0% to 33.3% saturation range.

3. The supernatant over the precipitate at 50% to 51% saturation with ammonium sulphate is relatively inactive in respect to both sparing and protective activities.

4. By selecting samples on the basis of the results of tests it is possible to obtain fractions of plasma potent in protective factor and weak or lacking in sparing factor; and vice versa.

5. The therapeutic value of fractions of plasma which are fortified in protective antibody as well as denatured in respect to sparing factor constitutes an interesting problem that requires further elucidation.

EFFECT OF CORTISONE ACETATE ON AN EXPERIMENTAL PURULENT INFECTIOUS ARTHRITIS OF WHITE MICE

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As a result of a search for bacteria in the human throat capable of producing joint lesions in laboratory animals, three strains of alpha type *Streptococcus* have been isolated which cause an experimental infection in albino rats and mice characterized by a high incidence of polyarthritis and lesions in the heart and kidneys (Friedlander, 1951).

The response in mice to intravenous inoculation of various doses of these organisms has been found to be highly uniform. The incidence of arthritis and mortality produced by a given dose can be predicted with a high degree of accuracy even with relatively few animals per group.

It was felt that this infection might be useful in detecting slight changes in resistance in groups of treated animals. These changes might be reflected in changes in mortality and in the incidence of arthritis produced by a given dose of the bacteria. The first compound tested on this infection was cortisone acetate.

METHOD

Female white mice (NIH general purpose strain) weighing 18 to 22 g were used throughout the experiments. As the work progressed it became evident that emphasis on random selection of groups and a plan of alternate selection during the inoculation period contributed to the uniformity of results.

All mice received an intravenous inoculum consisting of 0.2 or 0.3 ml of a suitable dilution of a 24 hour horse meat infusion broth culture of strain JST. Some groups then received the compound to be tested given subcutaneously once a day, some received physiological saline, some received the aqueous vehicle used to suspend the compound, and some received no treatment. The animals were generally treated for 5 days receiving the first dose the day before being infected with the test organism. The incidence of arthritis and mortality were usually recorded on the 3rd, 6th, 7th, 8th, 9th, and 10th days after infection.

Any animal showing visible swelling or impairment of movement of one or more joints was recorded as having arthritis. No attempt was made to grade the animals on the basis of the number of joints involved or the severity of the lesions.

RESULTS

Untreated mice

Since the mice were infected by injection of a suspension of live organisms in broth, it was felt desirable to determine whether or not the suspension changed in potency during the period of inoculation, which generally lasted about 80 minutes. One hundred mice were divided into groups of 10 each and injected intravenously in consecutive order with 0.2 ml of a suspension of strain JST containing approximately 100,000 viable organisms as determined by plate count. The mortality and incidence of arthritis in these groups was recorded on the 3rd and 6th day after infection. The results on the 6th day are recorded in table 1.

In addition to these figures we may add the results from 4 groups of control mice from experiments 3 and 4, table 3, where approximately similar doses of strain JST were used. The percent with

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TABLE 1.—*Mortality and incidence of arthritis in mice six days after intravenous inoculation of approximately 100,000 viable JST streptococci.*

Group	No. of animals	Dead	Survivors with arthritis	% Mortality + arthritis
1	10	1	1	20
2	10	1	0	10
3	10	0	0	0
4	10	1	0	10
5	10	0	0	0
6	10	0	2	20
7	10	0	2	20
8	10	0	2	20
9	10	0	1	10
10	10	0	1	10
Total	100	3	9	12

arthritis ranged from 5 to 12 indicating good uniformity.

The mortality and incidence of arthritis obtained with the inoculum used for these animals are suitable for experiments designed to show the effect of substances which are harmful to the mice. However, for experiments designed to show beneficial effects the potency of the inoculum must be increased so that the mortality and incidence of arthritis are increased.

For this purpose an inoculum of approximately 800,000 viable organisms was used. This was obtained by injecting the animals intravenously with 0.2 ml of a 1:80 dilution of a 24-hour broth culture of the test organism. One hundred mice were divided into groups of 10 each and injected in consecutive order with this dose. The results on the 6th day are recorded in table 2.

The two control groups of 24 mice each in experiment 5 (see table 3), which received an inoculum of approximately 500,000 viable organisms, showed an incidence of arthritis of 42% and 38% six days after infection, conforming with the above results.

An inoculum of approximately 100,000 viable streptococci produced arthritis in from none to two mice in each group of 10 tested. An inoculum of approximately 800,000 viable streptococci produced arthritis in from 3 to 7 mice in each group of 10 tested. The distribution of incidence in the groups follows the pattern expected as a result of chance variation in resistance of animals.

From these and many other similar experiments, it is evident that there is a direct relationship between the incidence of arthritis and mortality and the size of the inoculum as determined by plate count. A comparison of the results in groups injected at the beginning and at the end of the inoculation period recorded in tables 1 and 2 shows that the potency of the broth suspension used to infect the animals does not change appreciably during the 80-minute period and also indicates the uniformity of the response. These experiments furnished evidence that strain JST might be well suited for demonstration of statistically significant changes in response of uniformly infected groups of mice given various forms of treatment.

TABLE 2.—*Mortality and incidence of arthritis in mice 6 days after intravenous inoculation of approximately 800,000 viable JST streptococci.*

Group	No. of animals	Dead	Survivors with arthritis	% Mortality + arthritis
1	10	0	6	60
2	10	0	5	50
3	10	0	3	30
4	10	0	4	40
5	10	0	4	40
6	10	0	5	50
7	10	0	7	70
8	10	0	4	40
9	10	1	3	40
10	10	1	5	60
Total	100	2	46	48

Treatment with cortisone

The results of 5 experiments in which various doses of cortisone acetate were used are summarized in table 3. Six day-readings are given here for the sake of uniformity even though, in experiments 1, 3, and 4, later readings showed greater differences between cortisone treated animals and control animals.

TABLE 3.—*Mortality and incidence of arthritis in mice 6 days after inoculation with JST strain of* Streptococcus.

Exp. no.	Inoculum	No. of mice	Treatment	Dead	Survivors with arthritis	% Mortality +arthritis
1	960,000	20	0.020 g/kg cortisone	1	7	40*
	960,000	20	None	0	0	0
	780,000	20	0.010 g/kg cortisone	0	14	70*
	780,000	20	0.005 g/kg "	0	10	50
	780,000	20	None	0	6	30
	75,000	20	0.020 g/kg cortisone	2	8	50*
	75,000	20	0.010 g/kg "	0	4	20
	75,000	20	0.005 g/kg "	0	0	0
	75,000	20	0.0025 g/kg "	0	5	25
	75,000	20	0.2 ml saline	0	2	10
	75,000	20	None	0	1	5
4	135,000	23	0.025 g/kg cortisone	4	7	38*
	135,000	24	0.015 g/kg "	0	9	29†
	135,000	25	0.005 g/kg "	0	6	24
	135,000	23	0.2 ml saline	0	2	9
	135,000	25	None	0	3	12
	500,000	24	0.001 g/kg cortisone	0	11	46
	500,000	24	0.0005 g/kg "	0	7	29
	500,000	24	0.0001 g/kg "	0	9	38
	500,000	24	0.2 ml saline	1	9	42
	500,000	24	None	0	9	38

* $P = <0.01$.
† $P = <0.05$.

In experiments 1, 2, and 3 the groups were formed one at a time by selection of mice consecutively from stock cages. The unexpected irregularity of results in experiment 3 led to the conclusion that more emphasis should be placed on random selection of animals. Groups were formed thereafter by placing all the mice to be used in one experiment in a pool and then assigning each mouse to a group in a random manner. Since this method was adopted, marked irregularities in results have not been observed. It is also evident that in experiment 1, the incidence of arthritis and mortality are lower than was expected for the calculated inoculum used. Since both groups received the same infecting dose the comparison between the treated and untreated mice is valid despite the level of the response to the inoculum. In this experiment, on the 13th day after infection, three out of the 20 control animals had arthritis and none had died. In the cortisone treated group, three had died and 13 of the 17 survivors had arthritis.

From the results recorded in table 3 it is evident that statistically significant increases in incidence of arthritis plus mortality were obtained when 5 daily doses of 0.025, 0.020, and 0.015 g/kg of cortisone acetate were used. Doses of less than 0.015 g/kg given once a day for the same length of time produced increases which were suggestive of a harmful effect but not conclusive. Doses below 0.005 g/kg given once daily for 5 days produced no detectable effects with the small numbers of animals used in each group. The aqueous vehicle used by the manufacturer to suspend the cortisone was tested in subsequent experiments and found to have no influence on mortality or incidence of arthritis produced by this test organism. Desoxycorticosterone acetate in doses of 0.001 to .040 g/kg given once a day for 5 days also produced no change in incidence of arthritis or mortality. Animals treated with those two materials responded like untreated and saline treated control animals.

DISCUSSION

Evidence of lowered resistance in mice to bacterial infection following adminis-

tration of large doses of cortisone has been suggested from several sources. Antopol (1950) reported the appearance of granulomatous nodules in the liver, kidney, and spleen in 8 out of 36 mice receiving 6 to 9 daily injections of approximately 0.10 g/kg of cortisone acetate. From these lesions the author reported isolation of *Corynebacterium pseudotuberculosis murium*. The control mice showed no such lesions. The author expressed the view that the frequency of this infection in the treated animals might depend upon a loss of immunity produced by high doses of cortisone.

Ragan et al (1949) and Creditor et al (1950) have presented evidence to indicate that in rabbits and human beings therapeutic doses of ACTH and cortisone interfere with wound healing by suppressing the normal development of granulation tissue. Spain et al (1950) have observed a similar effect in mice. These authors also found that cortisone treated mice showed a retarded macrophage response to injection of carbon particles in India ink. The dose of cortisone used was approximately 0.1 g/kg per day.

Two more recent reports suggest lowered resistance of guinea pigs and white rats to two other infectious agents. Spain and Molomut (1950) found that cortisone treated guinea pigs showed more extensive lesions and greater spread of tuberculous infection than the control groups. Kuzell and Mankle (1950) found in cortisone treated rats an increase in mortality and incidence of arthritis produced by the L4 strain of pleuropneumonia-like organisms.

From our own observations of the effect of various doses of cortisone acetate on experimental streptococcus infection in mice we feel justified in concluding that doses of 0.015 g/kg and above, given under the conditions described, increase the mortality and incidence of arthritis in these animals significantly. There was no apparent difference in the distribution or gross appearance of the lesions. Excess cortisone apparently has a harmful effect on the defense mechanism of the mice against this infection. The interference with normal fibroblast and macrophage activity mentioned above (Spain et al, 1950) might account for this effect since these tissues are involved in the protection of the animal against such infections.

SUMMARY

1. The response in mice to infection with various doses of strain JST of alpha type *Streptococcus* has been found to be highly uniform when measured in terms of mortality plus incidence of arthritis.

2. Five to 7 days of treatment with cortisone acetate in doses of 0.015 g/kg and above have been shown to produce statistically significant increases in incidence of mortality plus arthritis produced by this organism in mice.

3. The increases in mortality plus incidence of arthritis are thought to be due to a lowered resistance of the mice resulting from treatment with cortisone acetate.

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THE IN VITRO AGGLUTINATION OF ERYTHROCYTE-FREE AVIAN PLASMODIA

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The specific in-vitro agglutination of erythrocytic malaria parasites by the serums of infected animals has been reported both for the simian (Eaton;[1] Ray et al[2]) and the avian (Manwell and Goldstein;[3] Zuckerman[4]) host. Attempts to demonstrate the production of such agglutinating antibodies in the rabbit by the injection of living or dead parasites have been hindered by the fact that the suspensions injected into the rabbit contained the stromata of infected and uninfected erythrocytes. This necessitated adsorption of the rabbit antiserums with the erythrocytic components. Such a procedure seemed to remove all the antibodies for the parasitized erythrocytes (Eaton and Coggeshall[5]). Zuckerman[4] working with chickens as a host for certain avian malarias reported that the "antibody in anti-gallinaceum hyperimmune serum was more efficiently absorbed by normal red cells than by an equivalent quantity of homologous parasitized cells." Though several explanations for the observations are possible, Eaton and Coggeshall's reasoning "that the parasites cannot be completely separated from the red cells of the monkey or other animals in whose blood they multiply" gives the most plausible support of the data herein presented. Hemolysis with water or saponin or even specific hemolytic antiserums does not accomplish this separation adequately, and for avian erythrocytes the erythrocytic nucleus is an added complicating factor.

It has since been shown (Stauber and Walker[6]) that it is possible to obtain suspensions of erythrocyte-free avian plasmodia. These suspensions are not devoid of erythrocytic components, but these components are fragmented and represent but a fraction of the suspended material. The formalinized suspensions remain stable and reactive over considerable periods of time. It is the object of this paper to present the evidence that the rabbit produces anti-malarial agglutinins both of a group-specific and of a species-specific nature detectable by these suspensions of erythrocyte-free plasmodia (Stauber et al[7]).

METHODS

Although various types of antigens were used for injection into the rabbits, most antigens were prepared by the saponin hemolysis, enzymatic digestion technique, which results in erythrocyte-free plasmodia. Aseptic precautions were taken at all steps except making the saponin solution. Most of the antigens were formalinized (0.2%),

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3. Manwell, R. D. and Goldstein, F. 1940, J. Exper. Med. **71**: 409–422.
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but a few were assumed to be sterile and injected without the addition of preservative. In all more than 50 antigens were prepared for injection and testing. Formalinized suspensions of free plasmodia were still capable of specific agglutination two years after preparation by antiserums adsorbed free from duck erythrocyte agglutinins. This stability of the antigen is in contrast to the findings of Eaton,[1] who found that 1% formalin destroys agglutinability of the *knowlesi*-parasitized simian red cells within 24 hours in the refrigerator.

Antiserums were prepared in 20 rabbits and 10 ducks. The most common procedure for the rabbits was to give the rabbit two series of injections, each series consisting of 4 injections of doubling amounts of antigen (e.g. 0.25, 0.5, 1.0 and 2.0 ml) with from one to two weeks between injection series. In almost all cases half the injected material was given subcutaneously and the other half intravenously. The antiserums were collected 7 to 14 days after the last injection. Antiserums were prepared using nonparasitized as well as plasmodia-infected duck erythrocytes or digests of them. Although the serums from normal rabbits (uninjected) were also studied, the presence or absence of normal agglutinins is not indicated, because all serums tested were first adsorbed with nonparasitized duck erythrocyte components. In the ducks, serums were obtained (1) after the crisis of blood induced infection, (2) after one or two injection series of the erythrocyte-free plasmodia as with the rabbit and (3) after both infections plus one or two injection series.

Three types of nonparasitized erythrocytic materials were used for adsorbing the antiserums: saponized enzymatic digest residue,[6] hemolyzed erythrocytes and freshly drawn and washed whole erythrocytes. The first adsorptions were made with suspensions of digest residue and hemolyzed erythrocytes. Only the last adsorptions were made with fresh erythrocytes. The usual procedure was to adsorb until agglutination could no longer be observed using nonparasitized materials as test antigens in a slide agglutination test. At least one adsorption involved overnight storage of the reacting materials in the refrigerator. Ordinarily less than 6 adsorptions were required with the serums of rabbits injected with parasitized antigens.

The slide agglutination test was performed with one drop each of the appropriate antigen dilution and of adsorbed and diluted antiserum. A saline control on the antigen was included in every run. After mixing by rotation, the slides were incubated at 37 C in a moist chamber, remixed and read at 5 and again at 15 minutes. Both macroscopic and microscopic observations were recorded. An agglutination recorded ++++ was easily visible macroscopically. Microscopically, only a couple of clumps were seen per medium power field (100×) with few or no free plasmodia between the large clumps. A + agglutination was barely detectable macroscopically but under the microscope showed many small clumps per field each containing several plasmodia. The antigens always showed a small amount of extraneous clumped material. It was easy to exclude these from a positive specific agglutination reaction.

A number of attempts were made to utilize photoelectric devices for a more objective determination of the agglutination end point but without developing a more sensitive test. Klett-Summerson Photometer, Beckman Spectrophotometer* and the Libby Photronreflectometer† were the instruments tried. In all cases the instruments were sensitive only in the range where the results were obvious with the unaided eye as in the ordinary tube agglutination tests which were tried. Since all these other tests were also extravagant in the use of antigens and antiserums and consumed more time in setting up and reading, the observations to be discussed were made from slide agglutination tests.

Test antigens were made up by volumetric dilutions of centrifugated packed free plasmodia in buffered saline (pH 7.0). A 1 to 5 or a 1 to 10 suspension made satisfactory test antigens, though other dilutions were sometimes used. In a typical antigen dilution study at low dilutions, in the region of antigen excess, the zone phenomenon was observed and at higher dilutions the reaction was read with greater difficulty. Interestingly enough, test antigens of *Plasmodium lophurae* (and *Plasmodium circumflexum* also) gave preparations which were easier to read for agglutination than did *Plasmodium cathemerium*. This seemed to be related to the light-absorbing qualities of the preparation. *P. lophurae* at equivalent dilutions makes a much darker preparation than *P. cathemerium*. This is believed to be the result of the difference in amount of malaria pigment in the two species. Consequently, a ++++ agglutination of *P. lophurae* is probably not entirely comparable with a ++++ reaction with *P. cathemerium*. The estimations are relative and best compared with others involving the same antigen.

* We appreciate the counsel and aid of Dr. H. N. Coy who ran these tests for us.

† We are indebted to Dr. A. A. Boyden for advice and assistance in the use of this instrument.

TABLE 1.—*Agglutination of erythrocyte-free suspensions of* P. lophurae* *by antiserums from rabbit or duck after the 6th adsorption of these serums with stroma and digest of nonparasitized duck erythrocytes.*

Antiserum number	Materials used for injection of animals	Antiserum dilution				
		1:2	1:10	1:20	1:40	1:80
Rabbit						
L1	*lophurae*—parasitized cell digest	+++	+++	+++	+	±
L2	*lophurae*—parasitized cell digest	+++	++	++	±	±
C1	*cathemerium*—parasitized cell digest	+++	++	++	+	±
U1	Nonparasitized cell digest	–	–			
UC1	Nonparasitized duck erythrocytes	–	–			
N1	No injections	–	–			
Saline		–	–	–	–	–
Duck						
L1	*lophurae*—parasitized cell	++±		+++	++	+
L2	*lophurae*—parasitized cell	–				
L3	*lophurae*—parasitized cell	±				
N1	No injections	–				
Saline		–		–	–	–

* Antigen washed, centrifuged and diluted 1:5 on basis of volume of sediment.

RESULTS

As might be expected, agglutinins were found in the serums of normal ducks and rabbits to the antigens used. No further discussion of these innate (natural) agglutinins will be given here, since very few observations were made on them and especially since those in the duck will be discussed extensively elsewhere (Trager et al[8]). Furthermore, to make the procedures comparable, even the duck antiserums in these studies were adsorbed with digests of uninfected and even infected erythrocytes. Since this constituted a dilution as well as treatment of the antiserums, only acquired agglutinins are discussed here. Some of the rabbit antiserums are still reactive after more than 4 years and are regularly used in class demonstrations of this phenomenon.

In table 1 are found the results of slide agglutination tests on a group of rabbit and duck antiserums after 6 adsorptions with stroma and digest residue from uninfected duck erythrocytes. The agglutinin titers under the conditions of the experiment are relatively low but are specifically related to the injection of parasitized material. The results with duck antiserums are less consistent, some ducks producing good titers, others almost none. The possible reasons for these deviations and their relationship to acute infection and relapse are described elsewhere.[8]

Cross agglutinations between *P. lophurae* antigen and anti-*cathemerium* serum (table 1) indicate the presence of group-specific agglutinins in the antiserums. This is further elaborated in another experiment (table 2), where two different antiserums, one prepared against *P. lophurae* and one against *P. cathemerium*, were compared. These antiserums were thrice adsorbed with uninfected duck erythrocyte material and aliquots of these partially adsorbed antiserums were then adsorbed 4 times more with test antigens prepared from *P. lophurae*, *P. cathemerium* and *P. circumflexum*. Each antiserum shows group-specific agglutinating properties but by adsorption can be shown to possess species-specific agglutinins as well. In the *cathemerium*-anti-*cathemerium* and *circumflexum*-anti-*cathemerium* comparisons there is a possible suggestion of a closer relationship between *cathemerium* and *circumflexum* than between *cathemerium* and *lophurae*. This difference is not believed to be significant, though further work on this point should be a profitable undertak-

8. Trager, W., Stauber, L. A. and Ben-Harel, S. In press.

TABLE 2.—*Agglutination of erythrocyte-free plasmodia by rabbit serums after repeated adsorptions with uninfected and plasmodial materials.*

Antigen	Antiserum dilution	Antiserum LI (anti-*lophurae*)					Antiserum CI (anti-*cathemerium*)				
		No. and kind of adsorptions					No. and kind of adsorptions				
		3x uninf. + 4x *loph.*	3x uninf. + 4x *cath.*	3x uninf. + 4x *circum.*	3x uninf.	Saline control	3x uninf. + 4x *loph.*	3x uninf. + 4x *cath.*	3x uninf. + 4x *circum.*	3x uninf.	Saline control
ophurae	Undil.	–	++	++	+++	–	–	–	–	++	–
	1:5	–	++	+	+++	–	–	–	–	+++	–
	1:10	–	±	±	++	–	–	–	–	++	–
	1:20	–	–	–	+±	–	–	–	–	+	–
	1:40	–	–	–	±	–	–	–	–	–	–
cathemerium	Undil.	–	–	–	++	–	+++	–	+++	++++	–
	1:5	–	–	–	–	–	+	–	+	+++	–
	1:10	–	–	–	–	–	+	–	±	++	–
	1:20	–	–	–	–	–	±	–	±	++	–
	1:40	–	–	–	–	–	–	–	–	+	–
circumflexum	Undil.	–	–	–	+++	–	++	–	–	++++	–
	1:5	–	–	–	±	–	±	–	–	+++	–
	1:10	–	–	–	–	–	–	–	–	++	–
	1:20	–	–	–	–	–	–	–	–	+	–
	1:40	–	–	–	–	–	–	–	–	–	–

ing. This is especially true, since on the basis of morphology as well as by course of infection in the duck, *circumflexum* more closely resembles *lophurae* (Manwell[9]). Although it is known that there is little or no cross immunity between *lophurae* and *cathemerium* (Wolfson[10] and our own unpublished observations) no studies have yet been reported comparing *circumflexum* with either of the other two.

Strain specificity within a species is a characteristic of infections with some of the malaria parasites. Whether this agglutination reaction will be successful in demonstrating strain specificity in vitro still awaits the necessary trials.

It should be noted that as observed early and repeatedly in this work, these suitably adsorbed anti-plasmodial serums do not agglutinate suspensions of infected duck erythrocytes. This is not considered at variance with Manwell and Goldstein's findings[3] with *P. circumflexum*, since the materials used and the details of procedure are so different. These results do confirm the reports of Eaton and Coggeshall[5] and Zuckerman[4] on the influence of adsorption with uninfected erythrocytes on subsequent agglutination tests using parasitized erythrocytes. These findings emphasize, however, the high concentrations of antibody necessary to effect agglutination of malaria parasites still surrounded by the stroma of the avian nucleated erythrocyte. It takes but slight dilution to abolish this phenomenon.

9. Manwell, R. D. 1943, Am. J. Hyg. **38**: 211–222.

10. Wolfson, F. 1945, Am. J. Hyg. **41**: 123–125.

SUMMARY

Injections of rabbits or ducks with duck erythrocytes parasitized with *Plasmodium lophurae* or *Plasmodium cathemerium* or with free plasmodia obtained from such parasitized erythrocytes give rise to the appearance of specific agglutinins in the serum. These agglutinins are demonstrated by the usual in-vitro agglutination tests, the slide agglutination test being the most advantageous procedure. The antimalarial agglutinins in the rabbit are properly demonstrated only after suitable adsorptions of the antiserums by nonparasitized erythrocyte material. By these and further adsorptions with homologous or heterologous plasmodial suspensions both group-specific and species-specific agglutinins have been demonstrated.

AGGLUTININS FOR *ESCHERICHIA COLI* IN SERUM AND IN FECAL EXTRACTS FROM HUMAN ENTERITIDES AND FROM HYPERIMMUNIZED AND EPINEPHRINE-STIMULATED RABBITS

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Harrison,[1] following the report of Burrows et al[2] concerning the occurrence of fecal agglutinins in experimental enteric cholera in guinea pigs, initiated a study of fecal agglutinins occurring in humans suffering with salmonellosis, shigellosis and chronic ulcerative colitis. He reported, as had Davies[3] and several Russian investigators,[4–6] that the demonstration of fecal agglutinins seemed an additional and valuable laboratory diagnostic tool, especially in the diagnosis of cases such as chronic ulcerative colitis where no etiologic agent was established. None of these authors has mentioned the occurrence of agglutinins for coliform bacilli in fecal extracts. It is with the incidence and possible significance of coproagglutinins directed against *Escherichia coli* that this report is primarily concerned.

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METHODS

Fecal extract.—Of the various methods described for preparing extracts from feces for serological determinations, that employed by Burrows[2] for comparing the potencies of serum and fecal globulin in μg of globulin per ml would seem to be the more acceptable. For the work presented here, however, ammonium sulfate fractionation of small samples from numerous dysenteric stools was impractical. Instead of such treatment, each fecal sample was diluted with an equal volume of 1:10,000 formalin in 0.85% saline.[7] The specimens were next frozen and thawed on an average of three times to lyse the leucocytes, then centrifuged at 3000 r.p.m. for 15 minutes and the supernatant decanted. This liquid constituted the fecal extract and was stored in the frozen state. For titration, dilutions in saline were prepared according to the conventional methods employed in carrying out routine bacteriological agglutinations.

Mediums employed.—The following mediums were employed for initial isolation: S. S. Agar (Difco), S. S. minus B Agar (the Difco formula without the Brilliant Green), Desoxycholate-Citrate Agar (Baltimore Biological Laboratory), E. M. B. (Difco) and a lactose agar plate containing 1.5% lactose, 2% proteose peptone (Difco), 0.5% sodium chloride and 0.0002% neutral red. Veal infusion agar was employed for characterizing colony types and for growing cells to be used as antigens. Numerous lots of S. S. Agar were found to be toxic for Shiga strains. *Shigella sonnei* phase II would not grow on

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S. S. Agar[8] nor would a number of strains of *Shigella paradysenteriae* W. In fact, no one of these mediums, with the exception of the plain lactose agar, would support the growth of all gram-negative bacilli being worked with at the time this study was carried out.

Antigens.—All coliform antigens employed were prepared as "H" and "O" antigens in the manner prescribed for the preparation of salmonella antigens by Edwards and Brunner.[9] Shigellae were carefully separated into their "phases"[10–12] and separate antigens prepared from each "phase." For the comparative agglutinin studies reported here, only shigella antigens prepared from the so-called "specific phase" stored in 0.3% formalin at pH 7.2 to 7.4 were used.

Selection of a representative Esch. coli *antigen.*—During the course of study of over 1500 fecal extracts from cases of acute bacillary dysentery on the islands of Kyushu and Honshu, Japan, it was observed that extracts which would agglutinate *Shigella* species from a given patient would frequently agglutinate strains of *Esch. coli*, and sometimes even *Aerobacter aerogenes* and *Proteus vulgaris*, from the same patient. By selecting a small number of these agglutinable coliform strains and preparing from them antigens and antiserums, it was possible to show by reciprocal absorption tests, using shigella antigens and antiserums and fecal extracts, that in the majority of cases the *coli* strains were agglutinated only by agglutinins specific for them. There were isolated, of course, a number of coliform bacilli which contained antigens common to certain *Shigella* species. This problem of cross agglutination between coliform bacilli and shigellas has been thoroughly investigated by Mackie[13] and our findings simply served to confirm and extend his observations. For the investigation of *coli* agglutinins in fecal extracts and patients' serums, it is obvious that only those coliform bacilli having no agglutinogens in common with *Salmonella* and *Shigella* species would be suitable. A group of such coliform organisms was assembled from a number of dysentery cases and an effort made to select from them one or two strains which would contain agglutinogens common to several antigenic types of *Esch. coli*. Since serums for the antigenic analysis of the *coli* group such as has been suggested by the work of Kaufman[14] were not available, the following method was employed for the selection of an *Esch. coli* strain containing agglutinogens representative of the *coli* group. Some 500 coliform bacilli, isolated from dysentery patients and shown to be agglutinable by their homologous fecal extracts in dilutions of 1:5 or greater, were used for the production of smooth antigens. A large number of these strains were then set up against homologous and heterologous fecal extracts. It was found that some strains were agglutinated only by their homologous extracts, some by one or two heterologous extracts and one strain, *Esch. coli* 97-C-K, was strongly agglutinated by extracts from over 50 different cases of bacillary dysentery. This suggested that of the coliform organisms tested *Esch. coli* 97-C-K, possessed the broadest *coli* antigenic pattern. It was further demonstrated that this particular organism was not agglutinated by any of the standard shigella or salmonella serums. *Esch. coli* 97-C-K was capable of selectively absorbing its "homologous" agglutinins from fecal extracts and from the patients' serums without appreciably altering the concentration of other agglutinins present, e.g. shigella agglutinins. However, the antiserum prepared against *Esch. coli* 97-C-K in rabbits did agglutinate in low titer the nonspecific "phases" of *Shig. sonnei*, *Shig. paradysenteriae* W and *Shig. paradysenteriae* V. Of the coliform organisms tested, then, *Esch. coli* 97-C-K seemed most suitable for investigating the presence of agglutinins for *Esch. coli* in the fecal extracts and serums from normal persons and persons suffering from gastrointestinal disorders.

8. Wheeler, K. M. and Mickle, F. L. 1945, Antigens of *Shigella sonnei*. J. Immunol. **51**: 257–267.
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14. Kaufman, F. 1947, The serology of the *coli* group. J. Immunol. **57** (1): 71–100.

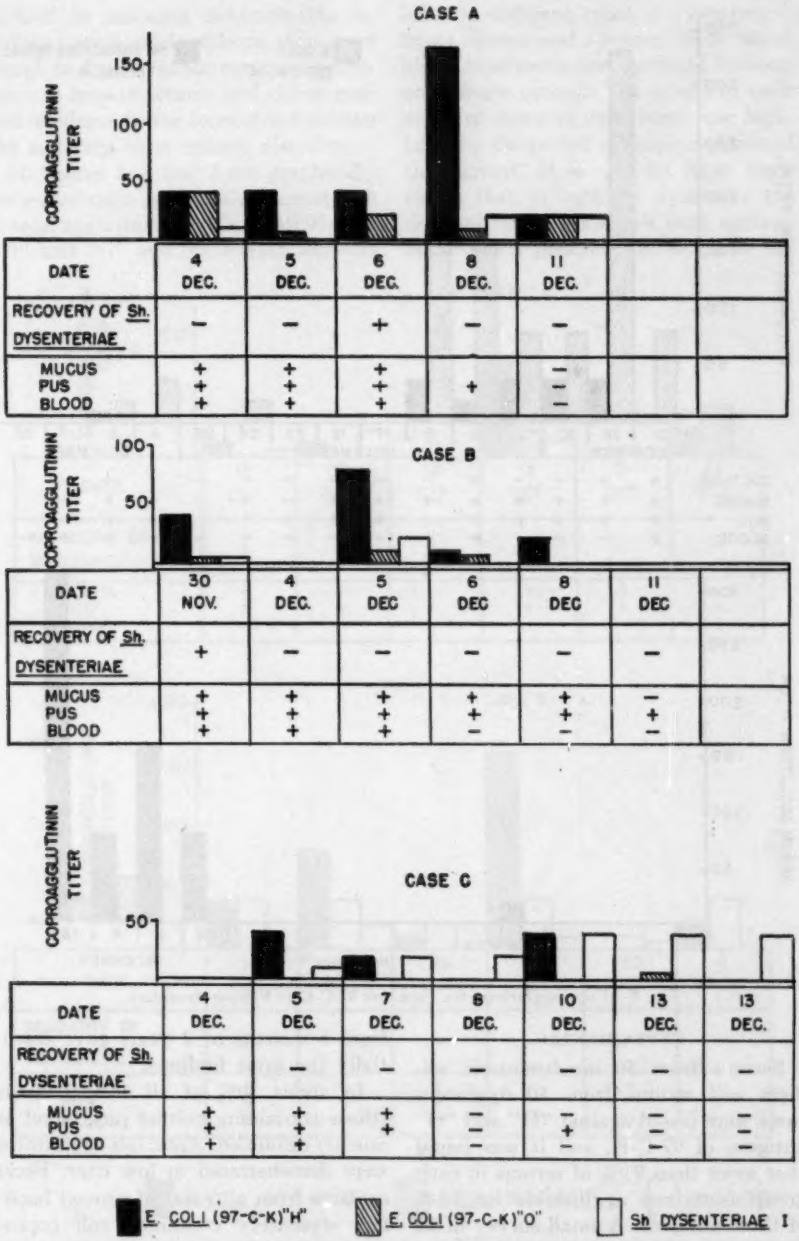

FIG. 1.—Coproagglutinins for *Esch. coli* 97-C-K in shiga dysentery.

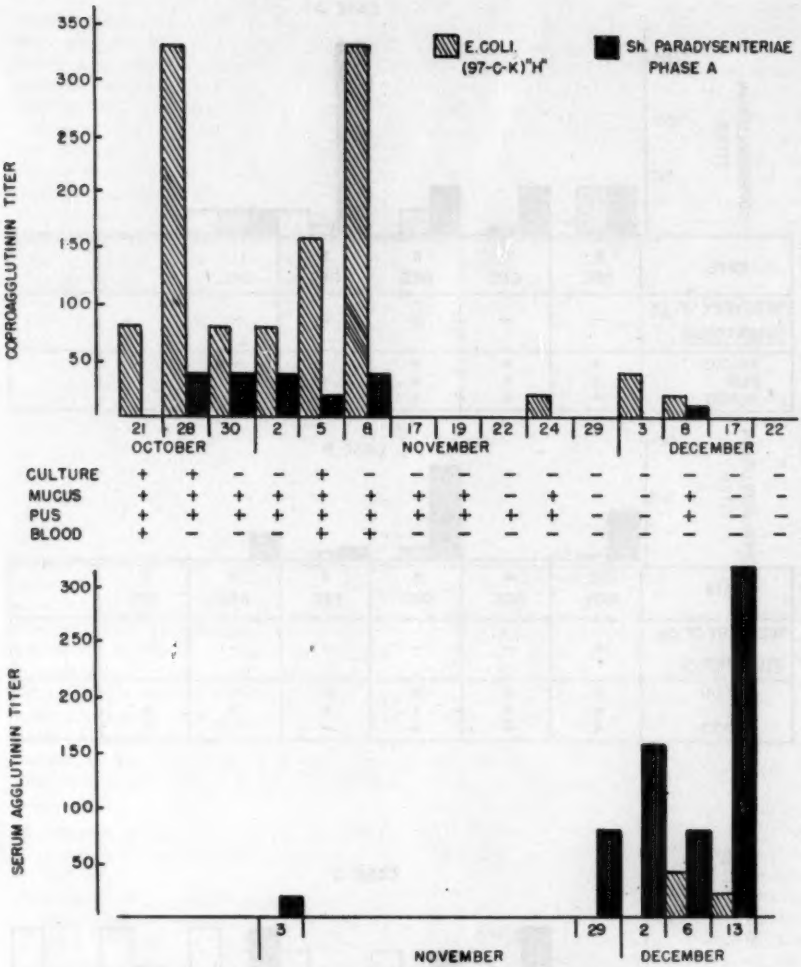
E.COLI (97-C-K)"H"
Sh. PARADYSENTERIAE PHASE A
COPROAGGLUTININ TITER
350
300
250
200
150
100
50
21 28 30 2 5 8 17 19 22 24 29 3 8 17 22
OCTOBER
NOVEMBER
DECEMBER
CULTURE + + - - + - - - - - - - - - -
MUCUS + + + + + + + + - + - - + - -
PUS + + + + + + + + + + - - + - -
BLOOD + - - - + + - - - - - - - - -
SERUM AGGLUTININ TITER
300
250
200
150
100
50
3 29 2 6 13
NOVEMBER
DECEMBER

FIG. 2.—Coproagglutinins for *Esch. coli* 97-C-K in Flexner dysentery.

EXPERIMENTAL

Serums from 50 nondysenteric soldiers and serums from 50 dysentery cases were tested against "H" and "O" antigens of 97-C-K, and it was found that more than 95% of serums in each group contained agglutinins for both of these antigens. A small survey of 15 serums from American infants ranging from 4 months to 2 years gave essentially the same findings.

In about 30% of all normal stools (those containing neither pus, blood or mucus) examined, *Esch. coli* agglutinins were demonstrated in low titer. Fecal extracts from all cases of proved bacillary dysentery contained coli coproagglutinins. Agglutinins for *Esch. coli*

97-C-K in amounts demonstrable by ordinary serological methods, then, were found to be of rather common occurrence in human serums and of less general incidence in the feces of individuals not suffering from enteric disorders.

In figures 1, 2 and 3 are graphically presented data on the relative strength of fecal agglutinins for *Esch. coli* 97-C-K "H" and "O" and for certain shigellas in three different types of dysentery—Shiga, Sonne and Flexner. Each dated block represents the serologic findings on a single extract. The extent of each series of extracts considered was regulated by the period of hospitalization of the patient. It is evident from these charts that in bacillary dysentery the rise and fall in *Esch. coli* fecal agglutinins roughly paralleled in each case the

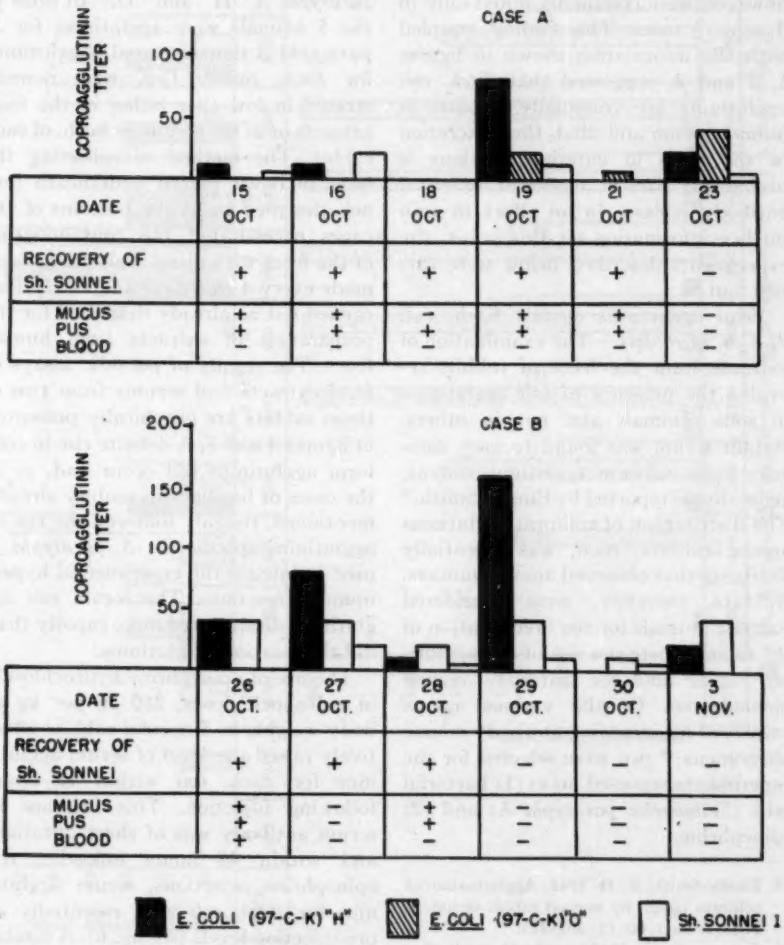

FIG. 3.—Coproagglutinins for *Esch. coli* 97-C-K in sonne dysentery.

rise and fall in fecal agglutinins for the recovered dysentery bacilli. The *coli* agglutinins were, in most extracts, present earlier and in higher concentration than were the shigella agglutinins. It has been previously stated that serums from normal and dysenteric individuals contained, in varying concentrations, agglutinins for *Esch. coli* 97-C-K. Significant levels of coli coproagglutinins, however, were commonly noted only in dysentery cases. This finding, coupled with the information shown in figures 1, 2 and 3, suggested that *Esch. coli* agglutinins are commonly present in human serum and that their excretion in the feces in enteric infections is probably a part of a general increased antibody release. In an effort to gain further information on this point, the experiments described below were carried out.

Fecal agglutinins against Esch. coli *97-C-K in rabbits.*—The examination of extracts from the feces of rabbits revealed the presence of *coli* agglutinins in some animals and not in others. Rabbit serum was found to vary similarly in its coliform agglutinin content, generally, as reported by Emslie-Smith.[15] The distribution of coliform agglutinins among rabbits, then, was essentially similar to that observed among humans. Rabbits, therefore, were considered suitable animals for the investigation of the relation between rise of *coli* agglutinin levels and the antibody release phenomenon. Of the various agents employed for initiating antibody release phenomena,[16] two were selected for the experiments reported here: (1) bacterial cells (*Salmonella paratyphi* A) and (2) epinephrine.

Five normal rabbits were selected for hyperimmunization with whole formalinized (0.3% formalin) cells of *S. paratyphi* A. The immunization protocol was that commonly employed in the production of diagnostic *Salmonella* antiserums.[9] All rabbits, prior to immunization, were checked for serum and fecal agglutinin levels against *Esch. coli* 97-C-K antigen "H" and "O" and *S. paratyphi* A "H" and "O." In none of the 5 animals were agglutinins for *S. paratyphi* A demonstrated. Agglutinins for *Esch. coli* 97-C-K were demonstrated in low titer either in the fecal extracts or in the serum, or both, of each rabbit. The method of collecting the feces in bowls placed underneath funnels designed to fit the bottoms of the cages necessitated the contamination of the feces with urine. Collections were made every 4 to 5 days and extractions carried out as already described for the preparation of extracts from human feces. The results of periodic assays of fecal extracts and serums from two of these rabbits are graphically presented in figures 4 and 5. A definite rise in coliform agglutinins did occur and, as in the cases of bacillary dysentery already mentioned, roughly followed the rise in agglutinins specific for *S. paratyphi* A used to initiate the experimental hyperimmune response. The serum *coli* agglutinins disappeared more rapidly than did the fecal *coli* agglutinins.

The use of epinephrine hydrochloride in sub-shock doses, 250 μg per kg of body weight, in 5 normal rabbits effectively raised the level of serum agglutinins for *Esch. coli* within 18 hours following injection. This increase in serum antibody was of short duration, and within 48 hours following the epinephrine injections, serum agglutinins for *Esch. coli* were essentially at pre-injection levels (see fig. 6). A similar effect was not reflected in fecal antibody levels. This undoubtedly was accounted

15. Emslie-Smith, A. H. 1948, Agglutination of coliform bacilli by normal rabbit serum. J. Path. & Bact. **60:** (2) 307–313.
16. Cannon, P. R. 1942, Antibody production and the anamnestic reaction. J. Lab. & Clin. Med. **28:** 127–139.

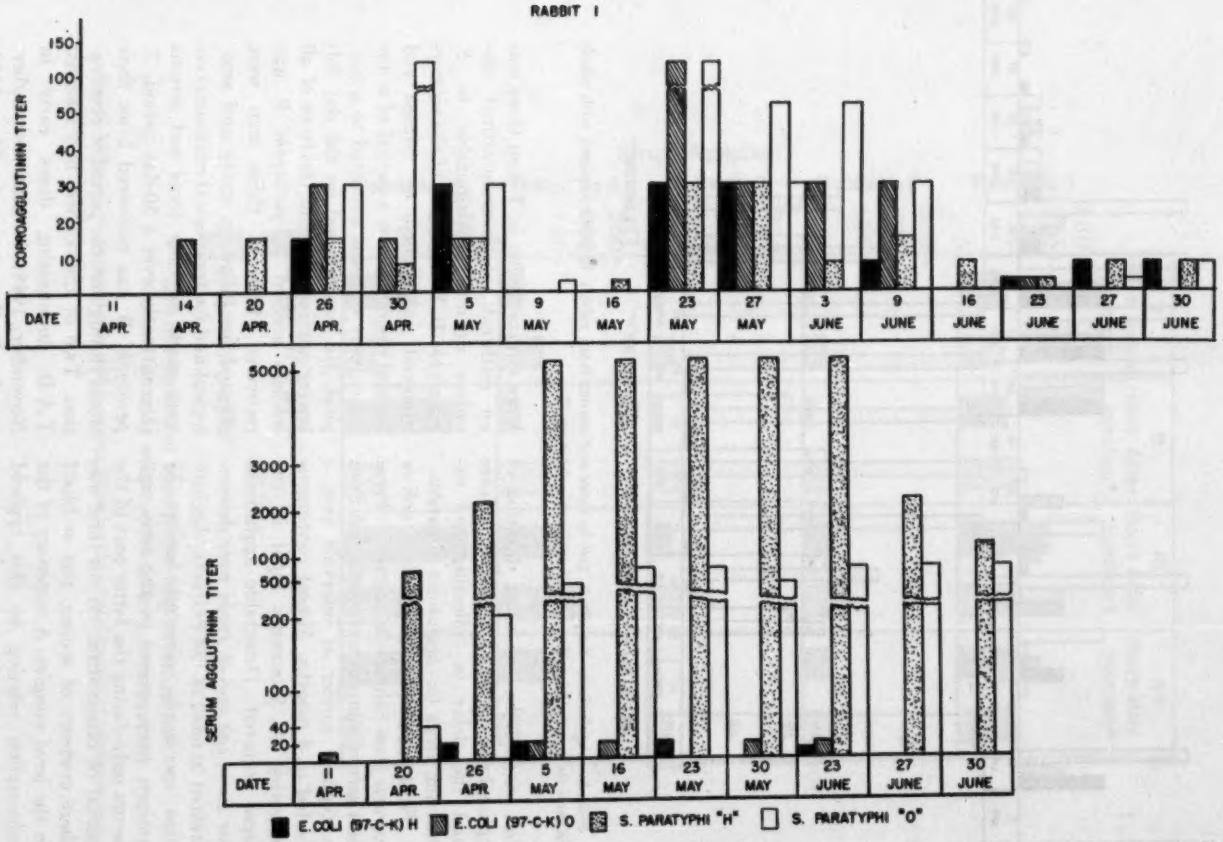

FIG. 4.—Agglutinins for *Esch. coli* in feces and serum from rabbits hyperimmunized with whole *Salmonella paratyphi* A (formolized antigen), rabbit no. 1.

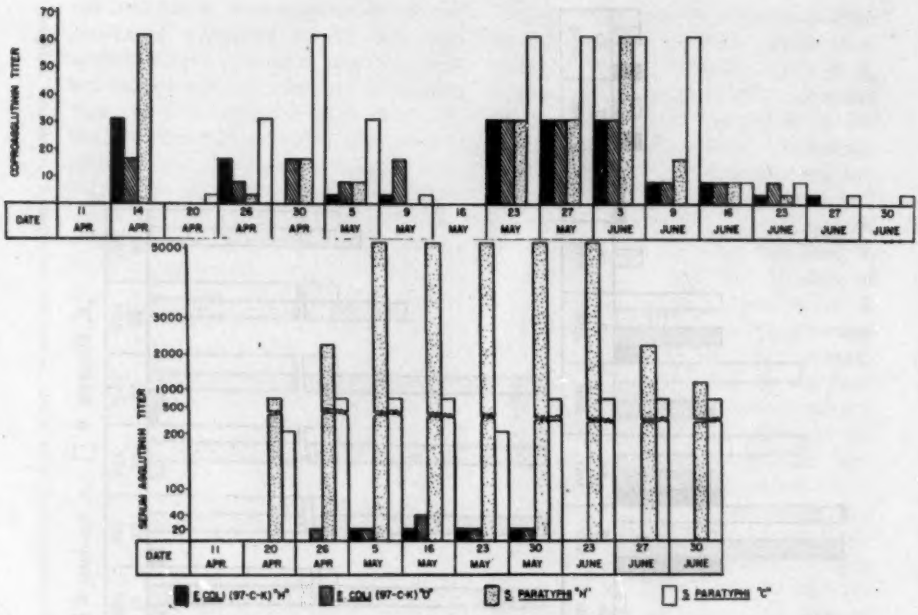

FIG. 5.—Agglutinins for *Esch. coli* in feces and serum from rabbit hyperimmunized with whole *Salmonella paratyphi* A (formolized antigen), rabbit no. 2.

for, in part, by the short duration of epinephrine induced antibody release and the delay in collecting and extracting feces for agglutinin titration.

Fecal agglutinins against Esch. coli *in extracts from* Salmonella *carriers.*—Fecal and serum samples were collected from a typhoid carrier at intervals over a period of 4 months. Almost continuous excretion of *Salmonella typhi* in the feces occurred. Detectable agglutinins for *Escn. coli* and *S. typhi* were demonstrated in most of the extracts. Agglutinins specifically directed against Vi antigen were present in the feces and serum only during the latter part of the period of observation. At no time was there evidence of mucus, pus or blood in the fecal samples. A summary of the information relating to this typhoid carrier comprises figure 7.

Following a Thanksgiving dinner in a large military billet in Tokyo there was an outbreak of gastrointestinal disorders definitely attributable to *S. paratyphi* B. While many of the soldiers involved complained of nausea and general discomfort for a period of a few hours, only one was admitted to a hospital. He was released on the day following admission. From the feces of all soldiers studied *S. paratyphi* B was recovered. Four of these men were selected for follow-up study and serological and bacteriological examinations were made on their feces and serums intermittently over a 50-day period. *S. paratyphi* B was recovered from their stools throughout the period of observation. Two of the soldiers had received T.A.B. immunizing doses early in November, 1948; the other two earlier, one in April and one in May, 1948. Their fecal extracts contained demon-

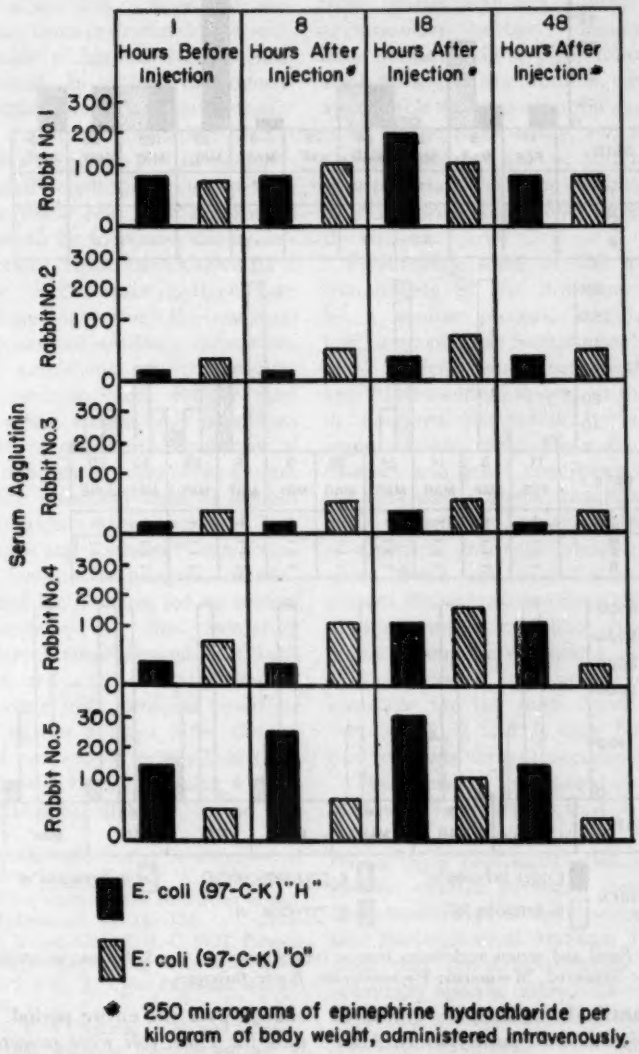

FIG. 6.—Rise in *coli* agglutinins in rabbit serums following the administration of subshock doses of epinephrine hydrochloride.

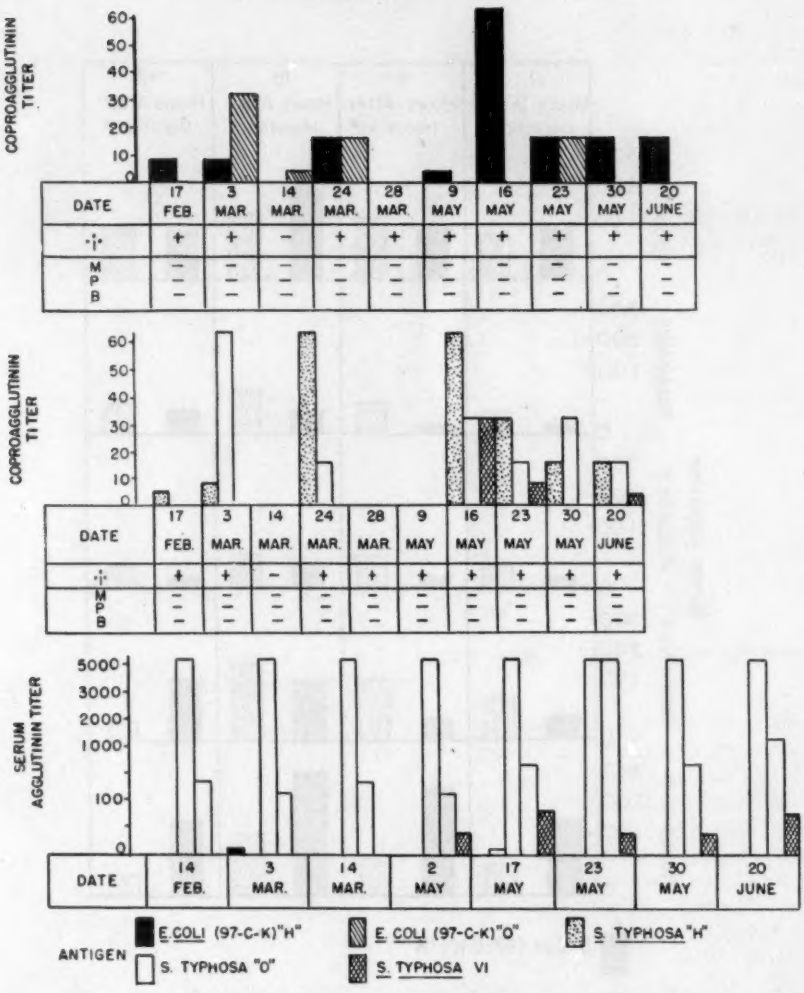

FIG. 7.—Fecal and serum agglutinins from a typhoid carrier. + = *S. typhosa* recovered; − = *S. typhosa* not recovered; M = mucus; P = leucocytes; B = erythrocytes.

strable antibody against *S. typhi*, *S. paratyphi* A and *S. paratyphi* B. After the first few days of observation, coproagglutinins were reactive in higher dilutions with *S. paratyphi* B flagellar antigens than with the flagellar antigens of either of the other two salmonellas. Serum agglutinins showed no significant change over the entire period. Agglutinins for *Esch. coli* were present in low concentration in the serum and in appreciably higher concentration in the feces.

DISCUSSION

The observations and experiments

recorded here indicate that serum, as well as fecal extracts of both man and rabbits, may contain demonstrable antibody capable of agglutinating certain coliform bacilli. In rabbits, the concentration of these antibodies was markedly increased during immunization with a heterologous organism and in epinephrine induced conditions approaching shock. In man, *coli* coproagglutinins were found to be increased during enteric infections. It has been known for a long time that certain antigens are capable of passing through the intestinal barrier to sites of antibody formation. Walzer,[17] employing the Prausnitz-Küstner reaction and Ratner and Gruehl,[18] using anaphylaxis in guinea pigs, have demonstrated passage of proteins from fish, egg and other sources through the intestinal walls of normal human subjects and guinea pigs. Lippard, Schloss and Johnson,[19] using complement fixation techniques, demonstrated that lactalbumin fed to normal infants appeared in the circulating blood. They further showed that antibodies directed against lactalbumin and against cow's milk proteins could be detected within 5 days after the ingestion of cow's milk for the first time. More recently, Hartley,[20] using 4 times recrystallized egg-albumin as an immunizing antigen and collodion particles coated with egg-albumin as an agglutinative antigen, demonstrated that normal guinea pigs, following a single feeding of egg-albumin, developed appreciable anti-egg-albumin agglutinin titers within three weeks, and that the average maximal titer so obtained was approximately the same as the average titer following parenteral injection of the antigen.

Presumably some of the antigenic components of the intestinal bacilli, by a similar process, stimulate the formation of their homologous agglutinins. Under normal conditions these agglutinins seldom appear in the bowel in amounts detectable by ordinary agglutination techniques. In enteric diseases and other conditions eliciting the so-called "alarm reaction" they occur in high titer, apparently as a part of a general antibody release phenomenon. Since *coli* agglutinogens were present throughout the duration of each of the enteric infections studied, it seems reasonable to assume that some of the increase in coproantibody responsible for the peak titers reached (see figs. 1, 2 and 3) may have been due to newly formed coproantibody.

The Russian investigators[4–6] and Harrison[1] have stated that in the examination of stool samples from enteritis cases coproantibodies against a specific enteric pathogen could often be demonstrated in the absence of positive bacteriological findings. They implied that the demonstration of these seemingly specific coproantibodies indicated the presence, prior or current, of homologous *Shigella* or *Salmonella*. By such reasoning these authors have accorded the demonstration of coproagglutinins, against *Salmonella* or *Shigella* strains, diagnostic significance. We feel, however, that the widespread presence of coproagglutinins against coli-

17. Walzer, M. 1927, Studies in absorption of proteins in human beings I. A simple direct method for studying the absorption of proteins. J. Immunol. **14**: 143–174.
18. Ratner, B. and Gruehl, H. L. 1934, Passage of native proteins through the normal gastrointestinal wall. J. Clin. Investigation, **13**: 517–532.
19. Lippard, V. W., Schloss, O. M. and Johnson, P. A. 1936, Immune reactions induced in infants by intestinal absorption of incompletely digested cow's milk protein. Am. J. Dis. Child. **51**: 562–574.
20. Hartley, G., Jr. 1942, The permeability of the gastrointestinal mucosa of guinea pigs to crystalline egg-albumin. J. Imumnol. **43**: 297–309.

form bacilli in individuals with enteric infections might greatly limit the diagnostic importance of fecal extracts found to agglutinate salmonella or shigella antigens. For, as has already been pointed out in the section on methods, there are a number of strains of coliform bacilli which contain antigenic components common to salmonellas[21,22] and/or shigellas.[13] The confusion the homologous agglutinins of such bacteria might cause in the serodiagnosis of enteric disorders certainly would seem to be considerable.

SUMMARY

The occurrence in low titer of coproagglutinins and serum agglutinins for strains of *Escherichia coli* in humans suffering with salmonellosis and/or shigellosis in normal subjects and in rabbits is reported. The strain employed, *Esch. coli* (97-C-K) was not agglutinated by standard salmonella or shigella antiserums.

In bacillary dysentery the rise and fall in coproagglutinins directed against *E. coli* 97-C-K roughly paralleled that of the agglutinins directed against the causal *Shigella*.

Evidence is presented which suggests that the mere demonstration in fecal samples of coproagglutinins directed against *Salmonella* or *Shigella* species cannot always be taken to indicate infection, past or present, with those organisms.

We are much indebted to Dr. William Burrows for his interest in this work and for providing us with copies of translations of the Russian investigations cited; and to Lt. Col. William D. Tigertt, M.C., for valuable and stimulating council throughout these investigations.

21. Wheeler, K. M., Stuart, C. A., Rustigian, R., and Borman, E. K. 1943, Salmonella antigens of coliform bacteria. J. Immunol. **47**: 59–66.
22 Bornstein, S. 1943, The state of the salmonella problem. J. Immunol. **46**: 439–496.

COPROAGGLUTININS IN ULCERATIVE COLITIS

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In a previous communication,[1] it was pointed out that serum and fecal antibodies directed against various coliform bacilli are of widespread occurrence in man and in rabbits. In this connection, mention was made of the observations of Mackie,[2] Luippold,[3] Bornstein,[4] ourselves[1] and others, that certain strains of coliform bacilli contain antigenic components common to certain *Shigella* and *Salmonella* species. Antibodies against such coliform organisms would be expected to agglutinate the *Shigella* or *Salmonella* strain to which they were serologically related. We, therefore, suggested that recognition of the existence of these cross reactions might appreciably limit the diagnostic value currently accorded coproagglutinins as indices to etiology[5–7] in enteric disorders where bacteriological findings are negative. Not until recently, however, have we been able to support this thesis with a practical example.

Lately, we have had an opportunity to study a case of chronic ulcerative colitis from whom neither salmonellas nor shigellas were isolated but whose coproantibodies were capable of agglutinating four or more species of *Shigella* and three species of *Salmonella*. A careful study of this case led to the isolation of a coliform bacillus (275-A-5) which could have been responsible for some of the antibodies mentioned. This coliform organism was agglutinated to high titer by both fecal extracts and by serum from the patient. Following the injection of small numbers of this organism the patient showed marked allergic reactions.

METHODS

The plating mediums employed and the methods for the preparation of antigens were those previously reported.[1] All salmonella- and shigella-typing serums, unless otherwise stated, were prepared by the United States Army Medical Department Research and Graduate School.

Biochemical tests.—All carbohydrates for the biochemical differentiation of mutants of 275-A-5 were C.P. grade and were used in 1% concentrations in tryptone broth containing 10 g of tryptone and 5 g of sodium chloride per L. Test inoculums consisted of 0.1 ml amounts of actively growing, plain tryptone broth cultures. The test broths were incubated for 14 to 20 days.

Preparation of the vaccine.—A freshly isolated strain of 275-A-5 was seeded on veal infusion agar and incubated for 12 hours. Harvests were made in 0.85% sodium chloride solution containing 0.5% phenol. The phenol killed suspension, nephalometrically standardized to contain

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1. Barksdale, W. L. and Ghoda, A. 1951, Agglutinins for *Escherichia coli* in serum and in fecal extracts from human enteritides and from hyperimmunized and epinephrine stimulated rabbits. J. Infect. Dis. **89**: 35–46.
2. Mackie, T. T. 1939, The specificity of the agglutinin reaction for *Shigella dysenteriae* II. J. Bact. **37**: 27–50.
3. Luippold, G. F. 1947, A paracolon organism antigenically related to Sachs Q1030 bacillus. Gastroenterology, **8**: 358–366.
4. Bornstein, S. 1943, The state of the salmonella problem. J. Immunol. **46**: 439–496.
5. Predpechensky, S. and Moroz, O. 1940, A method for early and rapid diagnosis of dysentery. Zh. Mikrobiol. Epidemiol. **7**: 3–11 (In Russian)
6. Yampolsky, S. M., Gorfunkel, D. M. and Aronina, V. B. 1944, Early diagnosis of dysentery in children. Pediatrya **5**: 13–18. (In Russian)
7. Harrison, P. E. 1948, Coproantibody excretion during enteric infections. Proc. of Meet., Soc. Am. Bact. **1**(1): 58–59.

100 million organsisms per ml, constituted the vaccine.

Toxicity tests.—Phenol killed suspensions of 275-A-5 were washed several times with saline and standardized to contain 100 million organisms per ml. Graded doses (0.5, 1.0 and 1.5 ml) of these washed cells were then inoculated intraperitoneally into mice. Two-tenths ml doses were introduced intracutaneously into the shaved ears of rabbits. The mice were observed for generalized reaction; the rabbits' ears for erythema and necrosis. With the highest amounts injected there were no reactions.

Case J.W.C.

The following information was taken from the Clinical abstract on case J.W.C.:

"This twenty-four-year old white soldier was admitted to the hospital 14 December 1948 with complaints of intermittent episodes of frequent loose stools containing blood and mucus. He had arrived in Japan in June of 1948 and shortly after noted blood and pus in his stools. No tenesmus or loss of weight, strength or appetite had been noted. At times his stools have become diarrheal, but usually he has noted from 1 to 6 semiformed stools daily—almost all stools have appeared 'dark red.' No fever or chills.

"His father is stated to have been discharged from the Army in 1917 because of bloody stools, pain in the back and otitis media.

"Patient was febrile throughout his hospital course. Only abnormal physical finding noted was on proctoscopic examination. On 21 December 1948 the mucus membrane of the sigmoid and rectum was diffusely reddened and bled easily. Many small, discrete areas were covered with densely adherent grayish exudate, varying in size from 0.25 to 1.5 cm. Barium studies showed the mucus membrane pattern in the terminal third of the descending colon and part of the sigmoid to appear thin and linear. The usual markings were absent. No evidence of ulceration noted. The radiological findings were suggestive but not diagnostic of an ulcerative colitis of the distal third of the descending colon and sigmoid.

"Grossly, the stool contained much mucopurulent material and dark red blood. Microscopically the stool showed many erythrocytes and leucocytes and a moderate number of macrophages. No cysts or ova noted. Stool culture negative for 'enteric pathogens and acid fast organisms.' These negative findings were consistently noted during his hospitalization.

"The patient was placed on sulfadiazine and a low residue diet plus belladonna and phenobarbital." (He apparently received sulfadiazine for six days with no improvement.) "On 5 February proctoscopic examination revealed hyperemic rectal and lower sigmoidal mucosa. Several small shallow ulcers without undermining were noted. The mucosa bled easily in several areas. Proctoscopy on 17 March showed some improvement in the mucosa."

Cultures carried out on a variety of mediums yielded none of the usual enteric pathogens. In extracts from the patient's feces, agglutinins were demonstrated for *Shigella* sp. Sachs Q902, *Shigella paradysenteriae*, *Shigella sonnei* I and II, *Shigella* sp. Sachs Q771, *Salmonella paratyphi* A, *S. paratyphi* B and *Salmonella typhi*. Further bacteriological examination of the feces, searching for both lactose positive and lactose negative colonies, yielded a coliform bacillus, 275-A-5, which was agglutinated in high dilutions by both the patient's serum and fecal extracts (see fig. 1). The component of 275-A-5 toward which this immune response was directed was labile to 100 C for 10 minutes. Use of the vaccine prepared from coliform 275-A-5 revealed that the patient was strongly sensitized to that organism. A dose of 0.05 ml (one-fourth of the amount producing no reaction in rabbits and one-thirtieth of the amount giving rise to no toxic symptoms in mice) of vaccine given intracutaneously on 14 February produced a delayed allergic reaction consisting of a region of induration and swelling 5 cm in diameter at the site of injection. Axillary nodes were swollen and the patient developed a severe headache and fever. A similar response was elicited by a second dose administered 5 days later. On 24 February a dose of one-twentieth the strength of the previous two produced no reaction. On 26 February the strength was increased to one-fifth the original dose. Moderate local reaction with slight

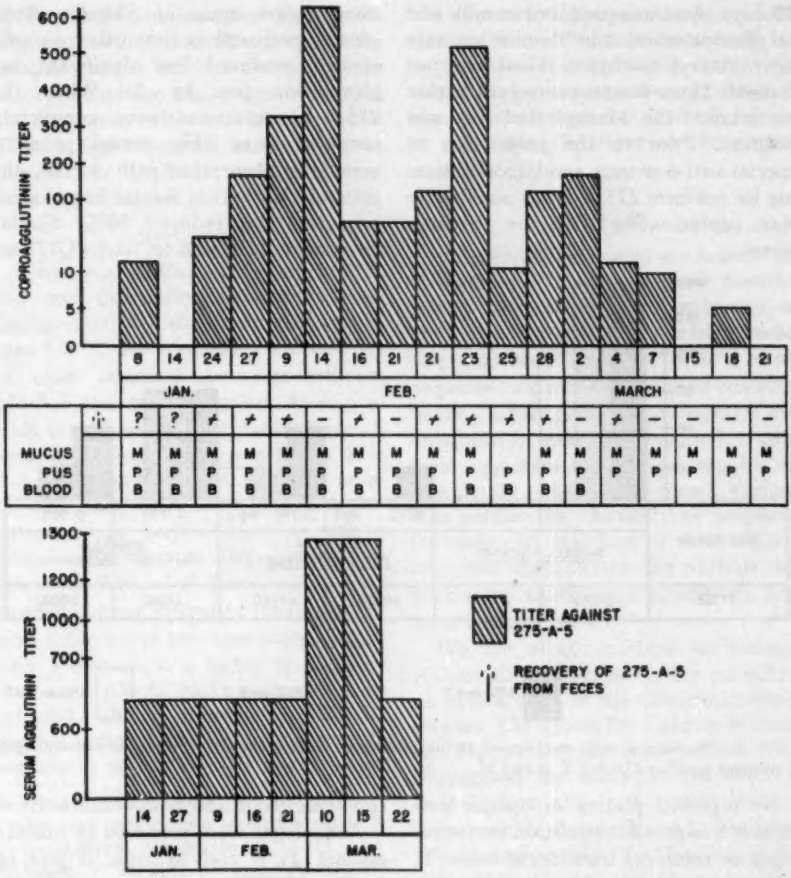

FIG. 1.—Immune response of case J. W. C. to coliform 275-A-5 as measured by serum and fecal agglutinins.

fever and headache resulted. A slight increase in the concentration of the inoculum on 28 February yielded a moderate sized wheal with no systemic manifestations. Administration of 0.05 ml of full strength vaccine on 2 March produced very severe local and systemic reactions. The study was terminated.

Coliform bacillus, 275-A-5

This organism was a short, nonmotile, gram-negative bacillus. When freshly isolated it grew as small, raised, opaque colonies on veal infusion agar. As will be shown later, these colonies gave rise to at least one distinct mutant. The initial isolates, i.e. the opaque colonies, will be referred to here as ϕ. The ϕ bacilli produced acid and gas within 48 hours from dextrose, trehalose, maltose, sucrose, lactose, raffinose, arabinose, rhamnose, salicin, mannitol, inisotol, sorbitol, and glycerol. No action on dulcitol or dextrin was evident after

20 days. Acid was produced in milk and an alkaline reaction in Simmon's citrate agar. Acetyl methyl carbinol was not formed. There was no proteolytic action on gelatin; the Methyl Red test was positive. Prior to the production of special anti-ϕ serums, serological screening for coliform 275-A-5 was possible by spot agglutinating with the patient's serum.

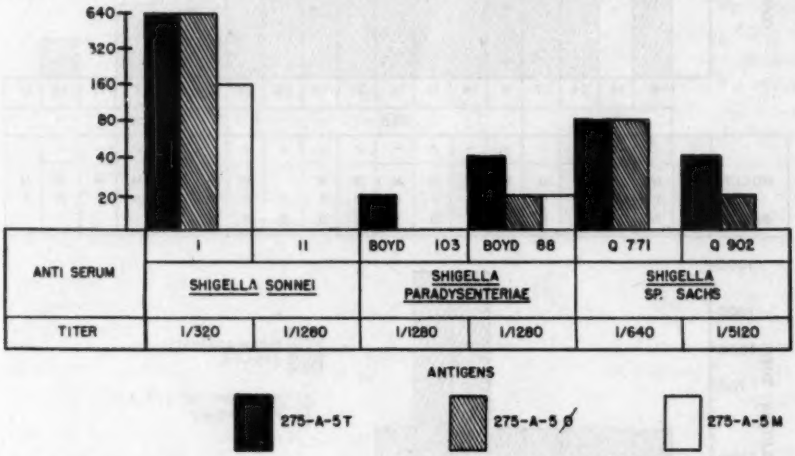

FIG. 2.—Serological cross reactions of certain absorbed, specific shigella antiserums with antigens of coliform bacillus 275-A-5 T, ϕ and M.

By repeated plating of opaque cultures it was possible to obtain two other types of colonies: translucent ones, T, and extremely mucoid ones, M. The T colonies were biochemically identical with their opaque parents; the M cells, however, rapidly produced acid and gas from dextrin. They made such viscid suspensions in broth in 18 hours that the tubes could be inverted with little or no flow of the medium. To obtain an even suspension of M cells for use as antigens, it was necessary to subject them to several washings with saline. Absorbed, specific diagnostic serum prepared against *Shig. sonnei*, phase I was found to agglutinate T, ϕ and M cells to twice the titer found with the homologous strain of *Shigella*. Antiserums prepared against other *Shigella* species produced less significant agglutination (see fig. 2). When the 275-A-5 agglutinins were completely removed from *Shig. sonnei* phase I serum by absorption with ϕ cells, the titer of that serum for its homologous organism was reduced 60%. Similar treatment of *Shigella* sp. Sachs Q771 serums resulted in a 50% reduction in titer.

Antiserums were prepared in rabbits against T, ϕ and M cells. Titers of 10,000, 20,000 and 5,000, respectively, were obtained. These antiserums, in dilutions of 1:640 and less, were found to bring about the agglutination of the specific and nonspecific phases of *Shig. sonnei*, *Shig. paradysenteriae*, types W, V and Boyd 103 and *Shigella* sp. Sachs Q771. Of the three serums, anti-ϕ was selected for absorption studies. Absorption with the ϕ antigen resulted in a complete removal of the ϕ homologous agglutinins as well as the shigella agglutinins. This indicated that the agglutination of the various *Shigella* species could be attributed to antibody directed

against some component of the ϕ cells and not to other antibody occurring naturally in the rabbit serums. A single absorption of the anti-ϕ serums carried out separately with each of the above *Shigella* types resulted in the elimination of the antibody responsible for their agglutination.

DISCUSSION

Serological studies with coliform 275-A-5 and the various *Shigella* species agglutinated by the fecal extracts of case J.W.C. have revealed a broad set of cross reactions between coliform 275-A-5 and those shigellas. It is possible that these coproagglutinins are all actually directed against coliform 275-A-5. It is also possible that one or more of them may have been formed in response to an earlier infection with a *Shigella*, for example *Shig. sonnei*. Felsen and Wolarsky[8] have noted that cases of chronic ulcerative colitis usually give a history of previous enteric infection. However, it is highly improbable that case J.W.C. had previously been infected with a number of *Shigella* species, some of which were endemic to regions in which he had never been.[9] The presence of the salmonella agglutinins mentioned may be accounted for on the basis of prior active immunization of the patient and the prevalence in him of a state of general antibody release at the time the fecal extracts were studied.

8. Felsen, J. and Wolarsky, W. 1947, Bacillary dysentery and chronic ulcerative colitis in World War II. Science, **106** (2721): 213.
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SUMMARY

A bacteriological and serological investigation of a case of enteric disorder falling into the general category of chronic ulcerative colitis has been made. Coproantibody studies revealed a broad response to numerous antigens common to the *Shigella* group of pathogens. Concomitantly, there was isolated a coliform organism which was capable of completely absorbing these various coproantibodies. Antiserums prepared in rabbits by injection of this coliform organism agglutinated the various shigellae for which coproantibodies had been demonstrated.

We are much indebted to Colonel Robert E. Blount, M.C., for providing us with a copy of the Clinical abstract on case J.W.C., to Lt. Colonel William D. Tigertt for his interest and help throughout the study, to the Medical Arts Section, the U. S. Army Medical Department Research and Graduate School and to Mr. Y. Matsudaira for the preparation of the figures used in these studies.

ANTAGONISM BY *GAFFKYA TETRAGENA* STRAINS

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To the authors' knowledge only one report has appeared concerning antagonism by members of the genus *Gaffkya*. A strain of *Micrococcus tetragena* was reported by Spousta[1] to be either weakly inhibitory to *Bacillus anthracis* or not inhibitory at all. This paper is concerned with an investigation of the occurrence of antagonism in strains of *Gaffkya tetragena* and the mechanism of the antagonistic action.

MATERIALS AND METHODS

Of the 6 strains of *G. tetragena* used in this study, 2 (6007-a and 6007-b) were acquired from the American Type Culture Collection; 2 (951 and 7501) from the National Collection of Type Cultures, London; one (Texas) from the Department of Bacteriology, University of Texas; and one (284) from a throat culture. The 17 test organisms listed in table 1 were from the stock culture collection of the Department of Plant Sciences of the University of Oklahoma. Morphological and biochemical properties of all *G. tetragena* strains and test species were found to conform satisfactorily to those in Bergey's Manual.[2]

For testing antagonistic properties, the *G. tetragena* strains were grown in Difco nutrient broth, pH 6.8, and tryptose phosphate broth (Difco Manual[3]), pH 6.9. Streaking was done with a standardized platinum loop of 3.2 mm outside diameter. Incubations were at 37 C.

EXPERIMENTAL

Antagonism in solid mediums.—Five-day cultures of the 6 *G. tetragena* strains in both nutrient broth and tryptose phosphate broth were brought to a temperature of 47 C and mixed with equal volumes of double strength nutri-

TABLE 1.—*Antagonism of* G. tetragena *strains by the "staled" agar technique.*

Test organism	Strains of *G. tetragena*											
	6007-A		6007-B		7501		951		Texas		284	
	NA	TPA	NA	TPA	NA	TPA	NA	TPA	NA	TPA	NA	TPA
Aerobacter aerogenes	0	4	2	2	4	3	2	3	2	4	4	4
Bacillus anthracis	0	4	0	0	2	0	4	2	1	4	0	0
Bacillus megatherium	0	2	1	0	0	1	2	3	1	4	0	2
Bacillus polymyxa	0	1	0	0	0	0	0	0	0	0	0	0
Bacillus subtilis	0	4	0	0	1	2	4	4	1	4	4	1
Escherichia coli var. *communior*	0	4	0	1	1	2	4	4	4	4	4	2
Escherichia coli var. *communis*	0	4	1	1	1	2	4	4	4	4	4	2
Micrococcus pyogenes var. *aureus*	0	4	0	0	0	1	0	1	1	4	0	0
Neisseria catarrhalis	0	0	0	0	2	0	2	0	2	2	0	0
Proteus vulgaris	0	4	1	1	1	2	4	4	1	4	4	3
Sarcina lutea	0	0	0	0	2	0	0	0	0	4	2	0
Serratia marcescens	0	4	1	2	1	2	2	1	4	4	1	2
Shigella dysenteriae	0	4	0	0	1	2	1	0	0	2	0	0
Shigella paradysenteriae	0	4	0	0	0	2	0	0	1	4	0	1
Shigella sonnei	0	4	0	0	0	2	0	0	0	4	0	1
Streptococcus faecalis	0	4	0	0	0	0	0	0	0	4	0	0
Streptococcus lactis	0	4	0	0	0	0	0	0	0	4	0	0

0 = complete inhibition, 1 = pronounced inhibition, 2 = moderate inhibition, 3 = slight inhibition, 4 = no inhibition (growth equal to control). NA = nutrient agar, TPA = tryptose phosphate agar.

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2. Breed, R. S., Murray, E. G. D. and Hitchens, A. P. 1948, Bergey's manual of determinative bacteriology, ed. 6, Baltimore, Williams and Wilkins Co.

3. Difco Laboratories, Inc. 1948, Manual of dehydrated culture media and reagents, ed. 8, Detroit.

ent agar or tryptose phosphate agar. The "staled" agar mediums were poured into petri dishes without undue delay. After solidification the 17 test organisms were streaked on the "staled" agar plates and on control plates prepared with distilled water in place of the culture filtrates. Results after 48 hours incubation (table 1) show that inhibition in nutrient agar ranged from a complete suppression of the growth of all 17 test species with strain 6007-a to moderate to complete inhibition of only 12 of the test species with strains 284 and 951. Inhibition in tryptose phosphate agar was decidedly less than that in nutrient agar with strains 6007-a and Texas, while the degree of antagonism observed on the two mediums appeared roughly equal with the remaining strains. Considering composite results with both mediums the least active strain (951) showed antagonism toward 13 of the 17 test species.

Filtrates.—Five-day cultures of the 6 *G. tetragena* strains in both nutrient broth and tryptose phosphate broth were filtered through Seitz and Mandler filters. Three of the 24 filtrates were lost. The remaining 21 were incubated 24 hours to check sterility, and 2 ml quantities were added to an equal volume of double strength nutrient broth in tubes. Since 4 of the filtrates of cultures grown in tryptose phosphate broth had a pH of 5.2 to 5.3, the pH of the final broth-filtrate mixtures from these strains was adjusted to 7.0 with sterile 0.1*N* sodium hydroxide. All other filtrates had pH values of 6.8 or higher.

The filtrate-broth mixtures were inoculated with a loopful of 24-hour broth cultures of the test species. A duplicate uninoculated set of filtrate-broth mixtures served as further sterility controls, and tubes prepared by adding sterile water instead of filtrates to double strength broth were used as growth controls. In a few cases some inhibition of *Sarcina lutea*, *Shigella dysenteriae* and *Shigella paradysenteriae* was noted at 12 hours of incubation, but at 24 hours no inhibition of any of the 17 test species could be detected.

Heat killing.—Five-day cultures of the 6 *G. tetragena* strains in both nutrient broth and tryptose phosphate broth were heated to 60 C for one hour and used as bases for the preparation of "staled" agar plates as above. Growth of all 17 test species on such plates was equal to that on control plates.

Killing with chloroform and toluene.—Despite the above failures to demonstrate an antibiotic substance, it was conceivable that a nonfilterable thermolabile substance might be involved. Therefore other methods of killing were employed.

Nutrient broth cultures of *G. tetragena* strains 6007-b, 7501, and 284 were treated with 0.5% chloroform for a time sufficient to render sterility control cultures negative. Residual chloroform was volatilized by continuous evacuation to approximately 1.5 cm of mercury with a "Hyvac" pump. The sterilized cultures were then used as bases for preparation of "staled" agar plates. Comparison of growth on such plates with that on control plates showed no inhibition of any of the 17 test species. When toluene was substituted for chloroform, similar results were obtained.

Agar barrier layers.—The possibility that a nonfilterable antibiotic substance capable of diffusion through agar might be involved was tested by a modification of the multiple agar layer method of Kelner.[4] "Staled" agar plates were prepared in the usual manner from cultures of *G. tetragena* 6007-b, 7501, and 284. After solidification, a barrier layer of

4. Kelner, A. 1948, A method for investigating large microbial populations for antibiotic activity. J. Bact. **56**: 157–162.

TABLE 2.—*Growth of test species on plates prepared from dilutions of a nutrient broth culture of* G. tetragena 6007-b.

Test organism	Undiluted	Dilution of *Gaffkya* culture				
		1:2	1:4	1:8	1:16	1:32
A. aerogenes	0	4	4	4	4	4
B. anthracis	0	2	4	4	4	4
B. megatherium	0	4	4	4	4	4
*B. polymyxa	0	0	0	0	4	4
B. subtilis	0	4	3	4	4	4
E. coli communior	0	4	3	4	4	4
E. coli communis	0	4	3	4	4	4
M. pyogenes aureus	0	1	1	1	3	4
N. catarrhalis	0	3	4	4	4	4
P. vulgaris	0	3	4	4	4	4
S. lutea	0	3	3	4	4	4
S. marcescens	0	4	4	4	4	4
Shig. dysenteriae	0	2	3	3	4	4
Shig. paradysenteriae	0	2	3	3	4	4
Shig. sonnei	0	2	3	3	4	4
S. faecalis	0	1	0	1	4	4
S. lactis	0	0	0	1	4	4

0 = complete inhibition, 1 = pronounced inhibition, 2 = moderate inhibition, 3 = slight inhibition, 4 = no inhibition (growth equal to control).
* Readings at 24 hours of incubation; all others at 12 hours.

approximately 15 ml of nutrient agar was poured onto the surface of each plate. Unglazed porcelain covers were employed to prevent undue accumulation of surface water and resulting spreading of growth. After solidification, the 17 test species were streaked on the surface of the barrier layers. Growth in all cases was equal to that on control plates.

Seeded plates of test species.—One ml of a 24-hour broth culture of each of the test organisms was used to seed a plate of nutrient agar. The resulting 17 plates were then streaked with *G. tetragena* 6007-b, 7501, and 284. No inhibition was noted along the lines of inoculation in any case.

Effect of dilution.—"Staled" agar plates were prepared from two fold dilutions of a nutrient broth culture of *G. tetragena* 6007-b and streaked with the 17 test species. After 12 or 24 hours of incubation, depending on the growth rate, comparison of the growth was made with that on nutrient agar control plates. A majority of the organisms was no longer inhibited at a dilution of 1:8 and none was inhibited at a dilution of 1:32 (table 2).

DISCUSSION

Instances of bacterial antagonism which presumably required the presence of living cells have been described by Stickelbroch,[5] Gundel and Kliewe,[6] Isabolinski and Sobolewa,[7] Neufeld and Kuhn,[8] Wynne,[9] and Bowling and Wynne.[10] The term "direct antagonism" signifying a close contact of living cells of the antagonist with those of the inhibited organism, was used by Neufeld and Kuhn[6] to describe such phenomena.

It is considered possible that the antagonism exerted by *G. tetragena* strains is also of the direct type. The virtual absence of activity of Berkefeld and Seitz filtrates indicates that at least the principal antagonistic action does not involve a filtrable antibiotic substance. Furthermore, inhibitory effects independent of living cells could not be demonstrated by use of heat-killed cultures, chloroform-killed cultures, toluene-killed cultures, "staled" agar with barrier layers, or plates seeded with the test organisms. The complete disappearance of antagonism on plates at dilutions of 1:32 or less in all cases tested is

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6. Gundel, M. and Kliewe, H. 1932, Experimentelle Untersuchungen ueber das Antagonistisch wirksame Prinzip der Coli gegenueber Milzbrandbazillen. Zentralbl. f. Bakt., I, Orig. **124**: 519–528.
7. Isabolinski, M. P. and Sobolewa, R. M. 1934, Ueber den Antagonismus der Bakterien. Zentralbl. f. Bakt. I, Orig. **133**: 107–110.
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9. Wynne, E. S. 1947, Antagonism by *Aerobacter* strains. J. Bact. **53**: 469–478.
10. Bowling, R. E. and Wynne, E. S. 1951, Studies on the mechanism of antagonism by *Aerobacter* strains. J. Infect. Dis. In press.

considered compatible with the occurrence of direct antagonism.

Though none of the above 7 lines of evidence would be convincing separately, it is felt that together they lend considerable weight to the hypothesis that antagonism by *G. tetragena* strains may depend on the action of living cells in fairly close contact with cells of the affected species. Recent work on the mechanism of antagonism of faster growing *Aerobacter* strains has indicated that "direct antagonism" may also be involved in this case (Wynne,[9] Bowling and Wynne[10]).

SUMMARY

Six strains of *Gaffkya tetragena* were tested for antagonistic action against 17 test organisms by means of the "staled" agar plate technique. Three of the strains were active against all the test species, and the least active strain inhibited 13 of the 17. Nutrient broth and agar were superior to tryptose phosphate broth and agar for demonstrating antagonism with 2 of the strains.

Heating broth cultures to 60 C for one hour destroyed the antagonistic power of all strains. Seitz and Mandlei filtrates were essentially inactive. With the three strains tested, evidence for a diffusible antibiotic substance could not be demonstrated by means of chloroform-killed cultures, toluene-killed cultures, agar barrier layers, or plates seeded with the test species and streaked with *G. tetragena*. Finally, inhibition of all 17 test species by broth cultures of *G. tetragena* 6007-b disappeared at dilutions of 1:32 or less.

It is felt that "direct antagonism" (Neufeld and Kuhn[8]) involving relatively close contact of living *G. tetragena* cells with those of the inhibited species is at present the most feasible explanation of the antagonism exerted by *G. tetragena* strains.

THE EFFECTS OF BRUCELLERGEN SKIN TEST ON BRUCELLA AGGLUTININ TITER IN PATIENTS WITH BRUCELLOSIS AND OTHER DISEASES

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The diagnosis of brucellosis is still beset with certain difficulties. The most reliable evidence is obtained from the demonstration of the brucella organism in the blood, bone marrow or other materials. However, cultural methods are not always available and do not yield positive results in all cases. Brucella agglutinins in titers above normal are by no means always present, and the demonstration of allergy to brucella organisms or brucellergen does not differentiate between active infection and previous exposure. Impressed with a fairly large number of patients presenting clinical evidence of chronic brucellosis who reacted positively to brucellergen but whose serum did not contain a significant titer of brucella agglutinins, a study was undertaken to determine the effects of brucellergen, administered intradermally, upon the titer of these antibodies. A discrepancy of opinion regarding the effects of such skin tests upon antibody formation is evident from a perusal of the literature. For example, Elton[1] observed a marked increase in agglutinin titer in subjects following the administration of a brucella vaccine. On the other hand, Kirby and Rantz[2] failed to find a significant rise in normal persons following skin tests with either brucellergen or brucella vaccine. Recently, Carpenter and associates[3] reported that brucella agglutinins appeared in 30% of 57 patients following a positive skin reaction to brucellergen and/or brucella vaccine, but did not develop in any individual with a negative intradermal test. Data collected between 1946 and 1949 on the effects of positive brucellergen skin tests upon the agglutinin titer in 34 patients suffering from probable brucellosis and in 85 subjects who did not have brucellosis and failed to react to brucellergen, are reported in this communication.

MATERIAL AND METHODS

The diagnosis of probable brucellosis was based on the following criteria: (1) History of contact with brucella-infected animals in a rural district and/or consumption of unpasteurized, unboiled dairy products obtained from these herds; (2) Clinical symptoms and signs included fatigability, sweating, weakness, fever, chills, muscular aches and pain, secondary anemia, splenomegaly. Every effort was made to rule out other diseases by means of blood counts, sedimentation rate determinations, roentgen-ray examinations, urinalysis and continued clinical observation. It is fully realized that a positive diagnosis can be made only on the basis of recovery of the causative agent from blood, bone marrow and other materials.

For the study on the effects of a positive intradermal brucellergen test on the agglutinin response, all patients suffering from brucellosis whose serums prior to skin testing agglutinated brucella in a dilution of 1:80 and above were excluded. This report is concerned with 34 patients suffering from probable brucellosis whose serums failed to agglutinate brucella in a significant titer and who reacted to brucellergen

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TABLE 1.—*Result of agglutination tests following brucellergen skin test in cases of probable brucellosis.*

Case	Brucella agglutination before skin test	Skin test	Brucella agglutination following skin test by:							Remarks
			2 to 4 days	5 to 7 days	8 to 10 days	11 to 13 days	14 to 19 days	20 to 31 days	Over 32 days	
A.K.	Neg.	4+						1:200		
H.Y.	Neg.	4+					Neg.	1:1280		
H.S.	Neg.	4+	Neg.				1:640			
J.P.	Neg.	2+	Neg.						1:80	
R.F.	Neg.	4+		Neg.				1:160		
R.M.	Neg.	4+		Neg.			1:40	1:160		
G.B.	Neg.	4+			1:640	1:320				+Blood Culture
C.F.	Neg.	4+	Neg.		1:40					
CL.F.	Neg.	4+			1:200					
M.Mc.	Neg.	4+				1:40	1:160			
C.W.	Neg.	4+		1:160						
E.B.	Neg.	2+			1:80				1:40	
E.D.	Neg.	4+					1:500			
C.M.	Neg.	2+	1:10				1:80			
K.M.	Neg.	3+			1:160					
O.J.	Neg.	5+				1:640				
H.C.	Neg.	4+			1:40		1:320	1:320		
C.R.	Neg.	4+	Neg.	Neg.			1:200			
F.B.	Neg.	4+				1:320				
T.T.	Neg.	3+		1:20	1:20			1:320		
L.H.	Neg.	4+					1:10			
J.S.	Neg.	4+	Neg.			1:320		1:40		
N.S.	Neg.	2+	1:160							
D.B.	1:10	5+			1:40			1:80	1:80	
O.	1:10	4+		1:10				1:320	1:320	
A.K.	1:10	4+		1:20				1:80		
E.S.	1:10	4+	1:10	1:80			1:320		1:320	
D.J.	1:20	4+		1:20				1:320		
F.R.	1:20	4+		1:40		1:160				
F.O.	1:20	2+			1:200					
S.	1:20	1+			1:320					
E.	1:20	4+				1:80		1:40		
E.E.	1:20	2+			1:320					
M.F.	1:40	5+		1:160		1:640		1:320		

as well as with 85 patients who did not have brucellosis and gave a negative brucellergen skin test.

Brucellergen was kindly made available by Dr. Huddleson to one of us (H.S.K.); 0.1 ml was injected into the skin of the flexor side of the left forearm. The test was read after 48 hours and graded according to Huddleson.[4]

The titer of brucella agglutinins was determined in the usual manner. Serial dilutions of the patient's serum (0.5 ml) were prepared and mixed with an equal amount of properly diluted, alcohol-treated suspension of brucella, supplied by the Division of Laboratories and Research of the New York State Department of Health. Parallel tests were performed and incubated at 55 C and 37 C, respectively. The resulting agglutination was read after 48 hours. The highest titer obtained at either temperature has been referred to below.

RESULTS

Data on the agglutinin titers of 34 patients probably suffering from brucellosis prior to and following skin testing with brucellergen as well as the reaction to this antigen, are summarized in table 1. It is evident that all patients of this group, whose agglutinin titer prior to skin testing was negative in a dilution of 1:10 or positive in a dilution of up to only 1:40, showed an increase in agglutinin titer following the intradermal test; 32 patients developed antibodies in a titer of 1:80 or above. It may be noted also that the maximal increase took place between the 8th and 22nd day in the majority of these cases.

In contrast, 35 children and 50 adults who suffered from disease other than brucellosis and who failed to react to brucellergen even after repeated (up to 4 times) administration did not develop brucella agglutinins in titers exceeding 1:40.

DISCUSSION

The data presented here confirm and extend the observations of Carpenter and associates.[3] It is evident that only 30% of the individuals studied by Carpenter et al, whose clinical diagnoses

4. Huddleson, I. F. 1943, Brucellosis in Man and Animals, New York, The Commonwealth Fund.

are not given and who presumably were not selected to include brucellosis cases exclusively, developed brucella agglutinins. On the other hand, an increase in the titer of these antibodies occurred in all 34 patients diagnosed as probably suffering from brucellosis included in this report and a rise in titer to 1:80 and above in 32 (96%) of these cases. The far higher figure obtained in the present series as compared to that of Carpenter is probably related to the fact that the patients included in the former group were suffering from probable brucellosis while those of Carpenter were not selected on that basis. It is conceivable that the ability to react to brucella antigen administered intracutaneously with the production of brucella agglutinins demonstrable in the circulating blood does not persist as long as skin hypersensitivity. Should this be so, the determination of the agglutinin response following brucella skin tests may aid in the diagnosis of relatively recent exposure to brucella and of recent or present brucella infection. The antibody response of patients suffering from probable brucellosis who were treated with the newer antibiotics (aureomycin, chloromycetin, terramycin alone or in conjunction with streptomycin) is being studied at the present time, in order to determine the duration of skin hypersensitivity to brucellergen and of the ability to react to such a positive skin test with the production of brucella agglutinins demonstrable in the serum or at the site of the skin test.

SUMMARY

Positive brucellergen skin test in 34 patients selected for this study on the basis of a clinical diagnosis of probable brucellosis and the absence of brucella agglutinins in the circulating blood in significant titer was followed by an increase in the titer of brucella agglutinins in all cases and by an increase to a titer of 1:80 or above in 32 (96%) patients. Such an increase was not observed in 85 patients who did not suffer from brucellosis and who did not react to brucellergen.

THE ACTION OF SOME WATER-SOLUBLE CARCINOGENS ON BACTERIA

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In accordance with concepts of comparative biology, several investigators have turned to microorganisms in their search for a tool with which to study the action of chemical carcinogens. The results of these investigations include reports that various carcinogens stimulate,[1–7] inhibit,[8–11] both stimulate and inhibit (depending on the concentration),[12,13] or exert no influence on the growth of various organisms.[14–17] Morphological changes have also been described.[5,8,10,11,18–20]

A somewhat similar situation exists among the investigations on microbial enzyme systems as studied with "resting" cells or with homogenates. A few reports of stimulation or inhibition of respiration (sometimes depending on the concentration)[10,21] and of no effects on respiration[14] have appeared. Some studies on yeasts indicate that the energy-yielding system of the yeasts is the likely site of the inhibitory action of carcinogens. Thus, cozymase was implicated by Pourbaix,[10] triose phosphate dehydrogenase and cocarboxylase by Kensler et al.[22,23]

Although tests in the present study were not made for the possible mutagenic capacity of carcinogens per se, it should be noted that such investigations have been made, and are, on the whole, still inconclusive.[17,24–28]

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2. Dodge, C. W., Dodge, B. S. and Johnson, G. S. 1941, Ann. Missouri Bot. Garden, **28**: 1–30.
3. Goldstein, S. 1937, Science, **86**: 176.
4. Hopper, S. H. and Clapp, D. B. 1939, J. Bact. **38**: 13–23.
5. Mottram, J. C. 1940, Nature, **145**: 185.
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Some of the work cited above has been criticized because of poor methods, poor choice of materials, impure cultures, etc.[18] The use of unstable suspensions or solutions of uncertain concentration has undoubtedly contributed to the discordant results that have been obtained. However, the apparent contradiction in the results of studies with microorganisms is also evident in studies with higher organisms.[29]

The primary purpose of the present investigation was to study the action of three water-soluble derivatives of polycyclic hydrocarbons, two of which are known carcinogens, on the growth and metabolism of bacteria. The results obtained indicate that, of the compounds studied, only the two carcinogenic derivatives exerted a definite inhibition of growth on certain bacteria in proportion to the concentration of carcinogen. In addition, evidence has been obtained that the two organisms studied in detail, *Lactobacillus* 46 and Pneumococcus type I, are worthy of more extensive work on the site of action of carcinogens, on factors which can prevent the action of carcinogens, and on properties of cells made resistant to carcinogen-inhibition.

MATERIALS AND METHODS

Hydrocarbon derivatives employed

Three water-soluble derivatives of polycyclic hydrocarbons were used in this work. The parent compounds are methylcholanthrene, 1,2,5,6-dibenzanthracene, and the noncarcinogenic anthracene. Two of the derivatives are carcinogenic per se,[30] although weaker than the parent compounds. The third is a noncarcinogenic hydrocarbon derivative used as a control compound. Methods for making the derivatives may be found in Warren's paper.[31] Briefly, the procedure consists of a Diels-Alder addition reaction in which maleic anhydride adds directly to the hydrocarbon to give a new anhydride; this is hydrolyzed to give the endosuccinic acid.

The anhydrides of the anthracene and methylcholanthrene endosuccinic acids* were first converted to the free acid as follows: A 40% potassium hydroxide solution was slightly heated with a small amount of the anhydride and subsequently diluted until very little or no precipitate remained. The solution was then filtered and the residue discarded. Approximately 10 *N* hydrochloric acid was added to the filtrate until the white endosuccinic acid precipitated. Excess hydrochloric acid was added and the solution placed in the refrigerator overnight. The precipitate was centrifuged, washed in cold double-distilled water three times, and dried in vacuo at 56 C for 6 hours.

The endosuccinic acids were put into stock solution as their sodium salts as follows: for anthracene endosuccinic acid, it was necessary only to heat a weighed amount of acid with a slight excess of a calculated amount of 0.1 *N* sodium hydroxide. Water was added to give a stock solution of 1 mg/ml. Solutions were prepared aseptically or sterilized by filtration. For methylcholanthrene endosuccinic acid, this procedure failed to bring any appreciable amount into solution. Therefore, a weighed amount of acid was dissolved easily in a few ml of acetone and added to a calculated amount of 0.1 *N* sodium hydroxide solution such that a concentration of about 50 μg/ml was obtained. The acetone was distilled under vacuum, and water further distilled off at a temperature never exceeding 55 C, until a concentration of about 60 μg/ml was reached. Several attempts to get an appreciably higher concentration for a stock solution always failed. Heating the solution much above 60 C resulted in a precipitate which could not be put back into solution. The stock solution was prepared aseptically or sterilized by filtration. No difference in biological activity was observed by either method. The anthracene endosuccinic acid was also put into solution by the acetone method for control purposes. Since no effect on microorganisms was observed with the control anthracene derivative thus prepared, it

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* Supplied by Edcan Co., South Norwalk, Conn.

was concluded that no residual acetone was responsible for the effects noted for the methylcholanthrene derivative. In the latter part of the work, Dr. Evelyn Witkin of the Cold Spring Harbor Laboratory generously supplied a sample of sodium methylcholanthrene endosuccinate. The acetone method was used to put it into solution. Sodium dibenzanthracene endosuccinate† went into solution easily by dissolving it directly in water. Clear stock solutions of 1000 μg/ml or 500 μg/ml were prepared aseptically or sterilized by filtration. A clear solution could be obtained after autoclaving at 10 lb for 15 minutes, if to the solution one drop of 0.07 *N* sodium hydroxide were added prior to autoclaving. Otherwise, a slight opalescence resulted upon autoclaving. However, a few trials showed very little difference in the biological activity of any of the solutions of the dibenzanthracene derivative. Nevertheless, in all experiments reported, only clear solutions, prepared aseptically or sterilized by filtration, were employed.‡ Because other suitable hydrocarbon derivatives could not be obtained, they were not used in this investigation. The compounds listed above were prepared and used in subdued light or darkness, since the parent hydrocarbons are known to give biologically inhibitory products upon prolonged exposure to ultraviolet light.[19,28]

Mediums

Two mediums were used in this work. The yeast extract medium (Medium I) was usually prepared in double strength and diluted for use. The final concentration of all ingredients was 0.5%. The medium consisted of Difco yeast extract, tryptone, KH_2PO_4, and glucose and was autoclaved at 10 lb for 20 minutes after pH adjustment so that a final pH of about 7.4 was obtained. Medium II was a casein hydrolysate-glucose—salts medium used elsewhere for vitamin studies[32] and was prepared for use as was Medium I.

† Kindly supplied by Dr. E. Boyland, Chester Beatty Cancer Research Institute, England.

‡ The melting points of the compounds (uncorrected) are as follows: anthracene endosuccinic acid, 244 to 246 C; methylcholanthrene endosuccinic acid, 115 to 116 C; sodium methylcholanthrene endosuccinate (supplied by Witkin), 184 to 185 C; sodium dibenzanthracene endosuccinate (supplied by Boyland), 269 to 270 C.

32. Boyland, E. and Boyland, M. E. 1934, Biochem. J. **28**: 244–256.
33. Koser, S. A. and Fisher, B. J. 1950, J. Dental Research, In press.

Growth measurements

Rates of growth and total growth were often both determined. Several different types of measurements of growth were made, depending on the experiments. In several experiments, it was found that a bacterial nitrogen determination was the best quantitative procedure to employ. Numbers of bacteria as determined by viable plate counts and pH measurements were also sometimes used as indices of growth.

Turbidity measurements.—Turbidity measurements were made in a Lumetron photoelectric colorimeter on cultures contained in 18×150 mm pyrex tubes previously matched to within 2% light transmission units. The blue filter was used with the almost colorless semisynthetic medium, while the orange filter was used with the yeast extract medium. Uninoculated tubes were set at 100% transmission.

Total nitrogen determinations.—The micro-Kjeldahl procedure described by Groman[34] was used without modification. Nitrogen determinations were always made on cells centrifuged and washed three times in cold saline.

Cultures, inoculations etc.

The cultures used were taken from the Curator's collection of the Department of Bacteriology and Parasitology, University of Chicago, except for two organisms. *Lactobacillus* 46 was obtained from Dr. S. A. Koser and has been tentatively identified as *Lactobacillus casei.*[33] Pneumococcus type I was originally obtained from a patient at Billings Hospital, University of Chicago, and has been maintained in the Curator's collection, Department of Bacteriology and Parasitology. Inoculations, unless otherwise specified, were made from 15- to 24-hour cultures, depending on the organism employed. Usually, it was about the time the culture had reached the peak of the growth curve. The inoculated tube contained a 1:10,000 dilution of the original culture as inoculum. Except for the yeast incubated at 30 C, all cultures were incubated at 37 C. Pneumococcus type I was carried in stock culture on blood agar, and transfer was made every three days. It was always adapted to the yeast extract medium before use by two or three passages through the medium. All other organisms used were kept on various mediums but passed through the yeast extract medium prior to being used for an experiment.

34. Groman, N. B. 1951, J. Infect. Dis. **88**: 126–150.
35. Orland, F. J. 1950, J. Infect. Dis. **86**: 63–80.

TABLE 1.—*The effect of AES, MES, and DBAS* on the growth of bacteria in a yeast extract medium.*

Organism	Age of culture in hours§	Percent light transmission†			
		0‡	AES (35 μg/ml)	MES (32 μg/ml)	DBAS (35 μg/ml)
Pneumococcus type I	14	36	37	100	100
	120	22	22	96	100
Pneumococcus type II	14	59	59	83	89
	120	34	34	80	81
Pneumococcus type III	14	77	79	83	87
	120	36	36	72	90
Streptococcus pyogenes	19	76	76	88	93
	120	56	57	68	89
Streptococcus salivarius	11	22	24	42	45
	120	15	15	38	38
Streptococcus mitis	10	39	40	38	
	72	36	36	51	
Staphylococcus aureus	10	25	24	62	
	72	16	16	24	
Vibrio cholerae	10	67	65	69	
	72	16	16	37	
Corynebacterium diphtheriae	14	85	86	90	
	72	17	17	23	
Lactobacillus 46	72	29	30	60	

* AES = sodium salt of anthracene endosuccinic acid; MES = sodium salt of methylcholanthrene endosuccinic acid; DBAS = sodium salt of dibenzanthracene endosuccinic acid.
† Higher percent transmission values denote less growth, with 100 the value of a clear tube.
‡ Control-medium, without added chemicals.
§ Only one of several periodic readings made during the early growth phases is tabled.

EXPERIMENTAL RESULTS*

Spectrum of bacteria

A study of the effect of the three hydrocarbon derivatives on a variety of microorganisms was undertaken first. Included in the spectrum of organisms examined were several species representative of the gram-positive and gram-negative group, and a yeast. One culture medium was chosen which could support adequate growth for all the organisms investigated. This was the yeast extract medium, Medium I, at pH 7.4. In a survey type of experiment of this sort, it was decided to accept turbidity readings as a measure of growth.

Only the turbidity readings of those organisms showing effects that were inhibitory or doubtful have been included in the table. All tubes showing no effects by any of the three compounds were kept for at least 72 hours before being discarded. They were: *Pseudomonas fluorescens*, *Proteus vulgaris* X19, *Salmonella typhi*, *Salmonella typhi-murium*, *Serratia marcescens*, *Aerobacter aerogenes*, *Escherichia coli*, *Klebsiella pneumoniae*, *Alcaligenes fecalis*, *Streptococcus fecalis*, *Streptococcus lactis*, *Bacillus megatherium*, *Clostridium sporogenes*, and the yeast *Saccharomyces cerevisiae*.

The results in table 1 indicate that only the carcinogens (DBAS and MES) and not the noncarcinogen (AES) exerted an inhibitory effect on either the rate of growth or total growth, as measured by maximum turbidity attained by certain bacteria. In no case was a stimulatory effect observed. The degree of growth inhibition varied with the organism studied, ranging from 100% with Pneumococcus type I to slight with *Corynebacterium diphtheriae*. Except for *Vibrio cholerae*, the only micro-

* For convenience, the following abbreviations will be observed for the three hydrocarbon derivatives described later: AES, for the sodium salt of anthracene endosuccinic acid; MES, for the sodium salt of methylcholanthrene endosuccinic acid; DBAS, for the sodium salt of dibenzanthracene endosuccinic acid.

organisms that were inhibited were gram-positive, although not all the gram-positive organisms were inhibited. *Streptococcus mitis* was not inhibited but showed lysis effects after maximum turbidity had been reached. *V. cholerae* occasionally showed inhibitions of the rate of growth as well as lysis in the presence of carcinogen. It is important to bear in mind that the results recorded here are valid only for the particular experimental conditions imposed. Data will be cited later which indicate that an organism unaffected by the carcinogens in one medium may be strongly inhibited in another (see *Lactobacillus* experiments). Because of this fact, and because *Esch. coli* had received much attention in the past in this type of study, a special effort was made to obtain inhibition of *Esch. coli* by using a simple synthetic medium (glucose and inorganic salts) in addition to Medium I. No effect was observed.

TABLE 2.—*The effect of graded concentrations of carcinogens on the growth of* Lactobacillus *46 in a semisynthetic medium.*

Age of culture in hours	Percent light transmission†											
	Experiment A						Experiment B					
	0‡	AES* (60 μg/ml)	MES* (5 μg/ml)	MES (15 μg/ml)	MES (22 μg/ml)	MES (31 μg/ml)	0‡	DBAS* (5 μg/ml)	DBAS (10 μg/ml)	DBAS (20 μg/ml)	DBAS (30 μg/ml)	DBAS (60 μg/ml)
12	93	92	94	94	93	95	92	91	94	97	96	100
14.5							80	84	86	96	96	99
15	73	72	76	80	85	88						
18	47	46	55	68	79	81						
20							36	44	55	88	89	98
21	23	21	43	55	70	73						
24	16	15	35	48	60	67						
36							17	31	48	58	68	92
38	12	12	27	43	54	66						
41	12	12	25	43	55	65						
60							13	31	48	58	68	92
Bacterial nitrogen values per 2 ml for 60 hr. readings							135 μg	50 μg	29 μg	20 μg	14.3 μg	5.5 μg

*, †, ‡ See table 1.

Two organisms of the group inhibited by the carcinogens were selected for detailed study. They are *Lactobacillus* 46 and Pneumococcus type I. The relation between optical density (= 2-log percent transmission) and bacterial nitrogen throughout the growth phases of the culture were determined for *Lactobacillus* 46 in the yeast extract medium (Medium I) and in the semisynthetic medium (Medium II). It was found that a straight line relationship exists in both cases, but with different slopes. The more sensitive relationship (a 1:1 relationship) was evident for the lactobacillus in Medium II. These data established that optical density (or percent transmission) is a sensitive measure of growth up to about 28% transmission in Medium I and to about 20% in Medium II for *Lactobacillus* 46.

Experiments with Lactobacillus *46*

The inhibition of the growth of Lactobacillus *46.*—The first experiments in this series were designed to study the effects of graded concentrations of carcinogens on the growth of *Lactobacillus* 46. Total nitrogen determinations were made on cells from two sets of tubes. Thus, the inhibition of growth as expressed in differences in turbidity readings was related in absolute terms to the total nitrogen content of the cells examined. MES and DBAS were tested in two separate experiments, but they are presented as experiments A and B in table 2.

The results of this experiment indicate that the carcinogens MES and

DBAS inhibit both the rate of growth and the maximum growth obtained by *Lactobacillus* 46 in Medium II. This is in sharp contrast to results obtained with the same carcinogens and *Lactobacillus* 46 in two yeast extract mediums (see later experiments). The inhibitions observed in Medium II are greater with increasing concentration of added inhibitor. In the range from 10 to 30 μg/ml of carcinogen, inhibition is approximately proportional to carcinogen concentration.

The influence of pH on the inhibitory action of MES and DBAS.—It was next decided to study the influence of the initial pH of the medium on the inhibitory action of the carcinogens. For this purpose the semisynthetic medium was prepared as usual, except that base was added aseptically to tubes so that pH values ranging from 6.9 to 7.8 were obtained. Lower values of pH were not chosen because of the insolubility of the carcinogens at an acid pH. The data obtained are presented in table 3 and show that inhibitions of growth were more effective at lower pH values.

TABLE 3.—*The effect of the initial pH of the medium§ on the inhibitory action of DBAS* on* Lactobacillus *46.*

Age of culture in hours	Percent light transmission†							
	pH 6.9		pH 7.2		pH 7.5		pH 7.8	
	0‡	DBAS (10 μg/ml)	0	DBAS (10 μg/ml)	0	DBAS (10 μg/ml)	0	DBAS (10 μg/ml)
15	47	93	54	96	46	96	53	76
61	13	87	13	77	13	69	14	46
Bacterial nitrogen values per 2 ml for 61 hr. readings	136 μg	6 μg	134 μg	11 μg	136 μg	16 μg	135 μg	21 μg

*, †, ‡ See table 1.
§ Medium employed was the semisynthetic medium.

Bacterial nitrogen determinations as well as turbidity readings bear out the fact that a lower initial pH in the medium increases the inhibitory power of DBAS. Turbidity data on the other carcinogen, MES, bear out the results of DBAS.

The influence of yeast extract plus phosphate on the inhibitory action of carcinogenic agents in Medium II.—Early growth experiments with *Lactobacillus* 46 in a yeast extract medium disclosed that the inhibitory action of the carcinogens could be modified, depending on the lot of yeast extract that was used in the medium.

It was found that the carcinogenic agents could exert little inhibition effect on *Lactobacillus* 46 if the bacterium was grown in Medium I made with yeast extract lot 402219. A small but significant effect was produced in the medium made from yeast extract lot 40715. Differences in turbidity readings with this lot of yeast extract were always of about the same order of magnitude from one experiment to another, and were found to represent significant differences in terms of viable plate counts and pH produced. These results suggested experiments to test whether the yeast extract lot 402219 could prevent carcinogen inhibition when it was added aseptically to the semisynthetic medium. Preliminary experimental results showed that the yeast extract could not prevent the inhibition by carcinogens when so added. It appeared, then, that some factor(s), when autoclaved with the yeast extract, must produce the active agent(s) that antagonized the action of the carcinogens. Various combinations of yeast extract with other ingredients of Medium I were prepared, autoclaved together to

TABLE 4.—*The prevention of DBAS* growth inhibition of* Lactobacillus *46 by the addition of solutions§ of autoclaved yeast extract plus phosphate.*‖

Age of culture in hours	Percent light transmission†															
		Combinations without phosphate							Combinations of yeast extract and phosphate							
		Solution 1 added		Solution 2 added		Solution 3 added		Solution 4 added	Solution 5 added		Solution 6 added		Solution 7 added	
	0‡	0	DBAS (10 μg/ml)	0	DBAS (10 μg/ml)	0	DBAS (10 μg/ml)	0	DBAS (10 μg/ml)	0	DBAS (10 μg/ml)	0	DBAS (10 μg/ml)	0	DBAS (10 μg/ml)
7						87	91	83	87	88	90	87	87	83	88
10.5						46	58	26	47	58	68	47	61	33	44
48						9	40	7	35	10	15	12	22	9	11
72	13	53	10	48	11	40	10	40	7	35	10	13	12	20	9	10
Bacterial nitrogen values per 3 ml for 72 hr. readings	135μg	30μg				180μg	80μg	184μg	90μg	193μg	181μg	195μg	186μg	198μg	195μg	

* † ‡ See table 1.

§ Various solutions prepared and autoclaved to final pH 7.4 were added aseptically to final concentrations of 0.5%. Without phosphate: solution 1, yeast extract; solution 2, yeast extract plus tryptone; solution 3, yeast extract plus glucose; solution 4, yeast extract plus tryptone plus glucose. With phosphate; solution 5, yeast extract plus phosphate; solution 6, yeast extract plus phosphate plus glucose; solution 7, yeast extract plus phosphate plus tryptone.

‖ Medium employed was the semisynthetic medium.

a final pH of 7.4, and added aseptically to the semisynthetic medium. The final concentration of all added ingredients was 0.5%, and the various ingredient combinations are listed in table 4. All chemicals were added to the medium prior to inoculation.

The data in table 4 indicate that only the solutions containing yeast extract and phosphate autoclaved together were effective in preventing the inhibition of total growth by the carcinogens. Although data were presented for DBAS only, similar data (in turbidity measurements) have been accumulated for MES. It may be concluded, therefore, that autoclaving certain yeast extract lots together with phosphate produces a factor(s) which can largely, and perhaps completely, prevent the inhibition of the total growth of *Lactobacillus* 46 by certain concentrations of two carcinogenic agents. Data are available, however, which show that a concentration of 45 μg/ml DBAS could again partially inhibit total growth in the presence of the preventing factor(s). Note that the best antagonizing action was effected by solution 7. Even here, however, the rate of growth was partially inhibited.

An experiment was next undertaken to determine whether the most effective solution, containing autoclaved yeast extract plus tryptone plus phosphate, could reverse as well as prevent the inhibitory action of the carcinogens. Tubes for the experiment were set up in the following manner: In the first set, control tubes and DBAS-containing (10 μg/ml) tubes were inoculated and placed in the incubator for 5 hours. Solution 7 was then added to both tubes to see whether the DBAS-antagonizing factor(s) was effective after the inoculum was exposed to the carcinogen for 5 hours. In the second set, control tubes and DBAS-containing (10 μg/ml) tubes were inoculated, and growth was meas-

ured until it was determined that the maximum inhibition produced by the DBAS had been reached (16 hours). Solution 7 was then added to both tubes (in a final concentration of the factors to 0.5%), and growth was again measured after 23 more hours. The third set of tubes was set up and treated similarly to the second set, except that solution 7 was added after 48 hours of incubation in the presence of the carcinogen.

The data in table 5, together with the data in previous tables, show that the factor(s) in solution 7 can reverse the inhibitory action of the carcinogens as well as prevent it. Note that the carcinogen-containing tubes in sets 2 and 3 show higher maximum growth than control tubes after the addition of solution 7. This is probably due to the fact that the yeast extract solution (solution 7) cannot stimulate cultures that have already grown out somewhat (the control cultures) but can still stimulate cultures held in check, so to speak, by the carcinogens (the DBAS-containing cultures).

TABLE 5.—*The ability of autoclaved yeast extract plus phosphate plus tryptone (solution 7) to reverse the growth inhibitory action of DBAS.**

Age of culture	Bacterial nitrogen per 2 ml					
	Set 1 Solution 7 added after 5 hr. incubation†		Set 2 Solution 7 added after 16 hr. incubation‡		Set 3 Solution 7 added after 48 hr. incubation‡	
	0§	DBAS (10 μg/ml)	0	DBAS (10 μg/ml)	0	DBAS (10 μg/ml)
48 hours	199 μg	194 μg	165 μg‖	188 μg	140 μg‖	175 μg
4 days						

* See table 1.
† Organisms had grown only slightly.
‡ Maximum inhibition at DBAS concentration of 10 μg/ml had been reached.
§ Control tubes—no inhibition.
‖ See text for explanation of low value of nitrogen.

The 48-hour results show the probable bacteriostatic character of the inhibition exerted by DBAS. If an irreversible killing effect were shown by the DBAS, it is unlikely that a reversing agent could be effective 48 hours after contact with the inhibitor. The question of whether carcinogens can kill even in concentrations of 300 μg/ml has been examined more thoroughly in experiments with the pneumococcus (see later section). Because of the facts presented regarding the inhibition shown by DBAS and the ability of a certain factor(s) to prevent and reverse the inhibition, consideration should be given to the idea that the inhibition by the carcinogens may be of the competitive type. Exact quantitative data to establish the truth or falsity of this suggestion have not yet been obtained. For competitive inhibition, the ratio of antagonist to inhibitor that is required to reverse the inhibition should be constant in growth experiments employing several concentrations of each.[36]

Besides solution 7, other simple and complex compounds, singly and in combination, were tried in an attempt to prevent the inhibition of the growth of *Lactobacillus* 46 by the carcinogens. All failed. The choice of most of the compounds was rationally based on what has been speculated by some authors to be the initial site of action of chemical carcinogens (e.g., SH systems). The list which follows contains compounds which were usually used in three different final concentrations up to a maximum of 1 mg/ml: cysteine, glutathione,

36. Work, T. S. and Work, E. 1948, The Basis of Chemotherapy, New York, Interscience Publishers Inc.

sodium thioglycollate, ATP, DPN, ATP plus DPN, casein hydrolysate, sodium nucleate, reticulogen, Tween 80, egg albumin, lecithin, lyophylized bovine serum, and AES. A combination of vitamins, purines and pyrimidines was also used. The factors included were: riboflavin, niacin, thiamine, biotin, pantothenic acid, folic acid, para-aminobenzoic acid, pyridoxamine, vitamin B_{12}, uracil, thymine, guanine, adenine, xanthine, and hypoxanthine.

All of the above compounds were tried before information about the phosphate solutions was obtained. It is possible that if some of the solutions above were autoclaved together with phosphate, they too would show reversing activity.

Microscopic examinations.—Microscopic examinations for fluorescence* were made on saline-washed cells after prolonged contact with all three hydrocarbon derivatives. Slides, containing only the hydrocarbon derivatives, were also examined. The latter all fluoresced yellow. The examination of control slides containing only *Lactobacillus* 46 cells revealed either no fluorescence or occasional fluorescence in a formless shape, as dust or a foreign body might exhibit. Those cells in contact with the noncarcinogen AES behaved similarly. The cells in contact with the carcinogens fluoresced yellow in the form of *Lactobacillus* 46 rods. Fluorescing cells could be subsequently located under ordinary light. The results above are interpreted to mean that the carcinogens adsorb on and/or penetrate living *Lactobacillus* 46 cells, and that the carcinogens still remain after three saline washings. An examination for morphological changes of *Lactobacillus* 46 in the presence of the carcinogens revealed no gross abnormalities.

Experiments with pneumococcus

Pneumococcus type I was the only organism of many that were investigated whose growth could be completely inhibited in concentrations of carcinogen below 50 μg/ml. For this and other reasons, this organism was chosen for intensive investigation. The results of the study form a basis for characterizing the action of the carcinogens on the pneumococcus, with the implication, of course, that the same principles may apply to other organisms. In this regard, it is well to remember that although the inhibition of the growth of *Lactobacillus* 46 never reached 100%, it approached complete inhibition quite closely in the semisynthetic medium.

The relation between optical density and bacterial nitrogen throughout the growth phases of Pneumococcus type I in Medium I was determined as for *Lactobacillus* 46. The data show that a straight line relationship exists for the plot of bacterial nitrogen vs optical density, and that the optical density (=2−log percent transmission) is a sensitive measure of growth up to a reading of about 30% transmission.

The effect of graded concentrations of carcinogens on the growth of Pneumococcus type I.—Medium I, the yeast extract medium, was used for the growth experiments with Pneumococcus type I. Graded concentrations of MES and DBAS were used in two experiments carried out weeks apart. Several compounds were used as controls. They were used to eliminate the possibility that the action of the water-soluble carcinogens was due to that part of the hydrocarbon derivative which was added on the hydrocarbon in the Diels-Alder condensation. The control compounds were sodium maleate, sodium succinate, and

* Mercury vapor lamp AH-4 was used and was housed in Spencer Housing #370. Blue filter was Corning 5112, and the ocular yellow filter was #348.

sodium malonate. In addition, AES was used as the noncarcinogenic hydrocarbon derivative control; this has previously been used as a control compound in studies with carcinogenic hydrocarbon derivatives. The control compounds were used in concentrations equimolar to 32 μg/ml MES. All failed to affect the growth of the pneumococcus. Data in table 6 indicate that the carcinogens inhibit the growth of Pneumococcus type I to a degree approximately proportional to the concentration of carcinogen.

TABLE 6.—*The effect of carcinogens on the growth of Pneumococcus type I in yeast extract medium.*§

Percent light transmission†							
Experiment A				Experiment B			
Chemical added (μg/ml)	Time in hours			Chemical added (μg/ml)	Time in hours		
	18	24	120		10	16	120
0‡	55	33	22	0‡	78	35	22
MES.* 0.6	67	32	23	DBAS.* 0.5	88	39	33
MES, 3.1	95	39	29	DBAS, 5.0	95	61	64
MES, 12.4	97	60	57	DBAS, 10.0	98	96	78
MES, 21.7	99	95	84	DBAS, 30.0	98	99	99
MES, 31.2	99	99	93	DBAS, 60.0	99	100	99

*, †, ‡ See table 1.
§ The following chemicals equimolar with 32 μg/ml MES failed to affect the growth of the pneumococcus: AES, sodium maleate, sodium malonate, and sodium succinate.

A partial lysis was indicated in the tubes containing the higher concentrations of carcinogens (in time intervals between 16 and 120 hours which are not tabled), but this has also been found to occur in the presence of NaN_3, NaF, $HgCl_2$, and in aging cultures with this pneumococcus. In the case of the latter, the colorimeter is not sensitive enough to show lysis as ordinarily measured in the machine, but bacterial nitrogen determinations do show it. Even after partial lysis, however, percent transmission values remain in a graded series somewhat proportional to carcinogen concentration.

Factors influencing the inhibitory action of the carcinogens.—Experiments were next undertaken to study the influence of various factors on the inhibitory action of the carcinogens. It was found that the inhibition of growth was greater at a more acid pH (between 7.0 and 8.0), as had been found for *Lactobacillus* 46. In addition, it was found that when younger organisms were used as the inoculum, inhibition by the carcinogens was greater. This is in line with what is known about the greater susceptibility of younger organisms to deleterious influences. Anaerobic conditions augmented slightly the inhibitory effect of the carcinogen, as evidenced by the fact that after 4 days of anaerobic incubation no growth was evident in concentrations that aerobically allowed some growth (to about 85 to 90% transmission) within 2 to 3 days. Further studies revealed that inhibition by the carcinogens was independent of the size of the inoculum, was not influenced by added catalase, and could not be prevented by the same factors that were tried for the lactobacillus. The significance of these combined results is evaluated later with the aid of other data.

Examination for morphological changes and adsorption of carcinogens

A microscopic examination of gram-stained slides for morphological abnormalities in the presence of the carcinogens showed no evidence of swollen forms or any other gross abnormal features. Examinations for fluorescence, made in the same manner as for *Lactobacillus* 46, disclosed similar results, except in one case. With Pneumococcus type I, some few cells that had been exposed to AES as well as to the carcinogens fluoresced yellow. Yet no effect of the compound on growth was observed in even more than twice the molar concentration of the carcinogens.

Metabolic experiments.—Metabolic studies were started in an effort to get more definite information concerning

the mode of action of the carcinogenic agents. Experiments were directed towards a study of the effect of the carcinogens on both the respiration and anaerobic glycolysis of the pneumococcus. The conventional procedures described by Umbreit et al[37] were employed. Oxygen consumption was measured directly, and anaerobic glycolysis was measured in terms of carbon dioxide released from a bicarbonate buffer. In respiration studies, it was found that not enough hydrogen peroxide accumulated to affect the results since catalase did not change the rates. No effect of the carcinogen DBAS (100 μg/ml) was observed on either the low respiration or the high anaerobic glycolysis of the pneumococcus, even if the carcinogen was preincubated with the cells up to 2½ hours before addition of glucose. To help eliminate the possibility that the carcinogen, under the conditions in the respirometer, might not readily penetrate the cells, partially disrupted cells (obtained by freezing and thawing 7 times) were employed. Microscopic examination revealed that there were cell fragments in the various fields to account for an approximate one-fourth of the destruction of the cells. In addition, cellular metabolism was reduced. In spite of these measures, no significant differences in metabolism could be detected between control and carcinogen-containing flasks. The concentration of 100 μg/ml DBAS was more than twice that previously found to inhibit growth completely. Since no effects were observed, it may be concluded that DBAS apparently exerts no effect on the principle energy-yielding system of the pneumococcus (the enzymes concerned in anaerobic glycolysis); nor does it appear to affect the enzymes concerned with oxygen consumption. It would seem, therefore, that the effect of DBAS is probably produced on the assimilation and/or the synthetic mechanism of the organism.

Survival experiments.—It seemed desirable to test whether the carcinogen could exert a direct killing effect in a concentration appreciably higher than that found to inhibit growth completely. A survival type of experiment was undertaken in which phosphate buffer-washed pneumococci were exposed to a final concentration of 300 μg/ml DBAS contained in a 0.1 *M* phosphate buffer of pH 7.35 at 29 C. Control cultures without carcinogen were run so as to provide a base line to which the death rate in the presence of carcinogen could be compared. For this purpose, a 15-hour culture of pneumococcus was centrifuged and washed twice in cold phosphate buffer and resuspended to the original medium volume. The suspension was divided in half. To one-half was added an equal volume of phosphate buffer at pH 7.35. This was the control suspension. To the second half was added an equal volume of phosphate buffer including DBAS so that the final concentration of DBAS was 300 μg/ml in the buffer. In this manner both suspensions initially contained the same number of organisms. Samples to serve for the zero hour determination in both suspensions were therefore taken only from the control suspension. Periodic samples for viable plate counts were later taken from both suspensions, within 20 to 30 minutes of each other.

The data in table 7 indicate that a concentration of 300 μg/ml DBAS exerts no significant killing effect on Pneumococcus type I in a phosphate buffer of pH 7.35. Other data are available that show similar results in a yeast extract medium at 25 C. Three hundred

37. Umbreit, W. W., Burris, R. H. and Stauffer, J. F. 1949, Manometric Techniques and Tissue Metabolism, Minneapolis, Burgess Publishing Co.

TABLE 7.—*The survival of Pneumococcus type I in the presence of DBAS.**

Without carcinogen		Carcinogen added	
Hours of exposure	Cells in 0.1 *M* phosphate buffer, pH 7.35	Hours of exposure	DBAS in 300 μg/ml. Cells in 0.1 *M* phosphate buffer, pH 7.35
	Counts in viable bacteria per ml		
0	180,000,000	0	180,000,000
3	125,000,000	3.3	100,000,000
6	79,000,000	6.5	81,000,000
11	600,000	11.5	500,000
14	200,000	14.5	143,000

* See table 1.

μg/ml DBAS is more than 7 times the concentration which can completely prevent the growth of pneumococcus in a yeast extract medium. The results of this experiment plus those of the previous experiments in the respirometer lead to the conclusion that within the considerable range of concentrations tried, the carcinogens probably act in a bacteriostatic manner rather than by killing outright.

Comparative action of carcinogens and anionic surface-active agents.—Because the carcinogens resemble the anionic surface—active agents in general structure (large hydrophobic anion and small hydrophilic cation), an attempt was made to differentiate the action of the carcinogens from the action of two well known anionic agents, sodium dodecyl sulfate (SDS), and sodium taurocholate (ST). It was found that bovine serum completely prevented the inhibitory action of the detergents in concentrations of 30 μg/ml for SDS and 50 μg/ml for ST. The inhibitory action of the anionic agents was depressed compared to the control cultures in the presence of a larger inoculum and in the presence of dead pneumococci. These results are in line with what is known of surface-active agent antagonisms.[38] The results are in contrast, however, to those found for the carcinogens. Bovine serum did not prevent the inhibitory action of the carcinogens; nor did the size of the inoculum or dead pneumococci affect carcinogen inhibition. This is therefore taken as presumptive evidence for the belief that the inhibitory action of the water-soluble carcinogens is different in some manner from the action of at least some anionic surface-active agents. This fact has also been observed in a different type of experiment[39] on frog tissue. The fact that the carcinogens also exerted no effect on respiration or anaerobic glycolysis is a different result from what has been observed elsewhere with various surface-active agents,[40,41] and is taken as additional evidence that the effects of the carcinogens probably cannot be attributed solely to the surface-active properties resulting from their general structural resemblance to surface-active agents.

Experiments with MES-resistant pneumococci

The study of strains of organisms made resistant to drugs has sometimes contributed to knowledge regarding the mode of action of the agents or to the comparative biochemistry of two closely related yet different strains. This general approach was also attempted with the carcinogens.

Development of an MES-resistant strain.—During the progress of this work, a strain of Pneumococcus type I was obtained whose resistance to the carcinogenic agent MES was increased several fold over that of a normal susceptible strain. The resistant strain was

38. Putnam, F. W. 1948, Advances in Protein Chem. **4**: 79–122.

39. Briggs, J. B. and Briggs, R. W. 1943, Cancer Research, **3**: 1–12.

40. Baker, Z., Harrison, R. W. and Miller, B. F. 1941, J. Exper. Med. **73**: 249–271.

41. Knox, W. E., Auerbach, V. H., Zarudnaya, K. and Spirtes, M. 1949, J. Bact. **58**: 443–452.

developed by inoculating the normal organism through many transfers in a yeast extract medium containing low concentrations of carcinogen. After 2 transfers per 24 hours for a period of a week, the concentration of carcinogen was raised slightly. This was carried on for a period of 7 weeks until the strain was "completely resistant" to 12 μg/ml of MES. A strain is here arbitrarily defined as resistant to a particular concentration of carcinogen when either its rate of growth or maximum growth are considerably less inhibited than the control strain. The degree of resistance is determined by comparing the highest concentrations of carcinogen in which a susceptible and resistant strain will grow without inhibition of their respective rates of growth; or, alternatively, by comparing the maximum growth attained in the presence of the inhibitor with that attained in control strains. These definitions have been made in place of others that use the all-or-none criterion of growth or no growth for reasons peculiar to the use of MES. DBAS, because it could completely inhibit the growth of the pneumococcus, would have been the compound of choice, but it could not be obtained at the time the experiments were started.

During the period of developing the resistant strain, a companion susceptible strain was carried through the same number of transfers through the same medium but without the carcinogen. It was unexpectedly found that just by passing the strain in this manner, its resistance to MES was increased. Compared to the original susceptible strain carried on blood agar, which was susceptible to 0.6 μg/ml of MES, the new control strain became susceptible at concentrations just greater than 3 μg/ml, a 5-fold increase over the original. The resistant strain became susceptible at a concentration just over 12 μg/ml MES, a 4-fold increase over the new control strain. The data to be presented show comparisons only between the MES-resistant strain and the new control strain (henceforth called "control strain"). Comparisons between control and MES-resistant strains were made with respect to their fermenting abilities, their rates of growth in yeast extract medium, and their cross resistance to the carcinogen DBAS. Before any comparisons were made, the MES-resistant strain was passed 7 times through medium free of carcinogen.

Biochemical fermentations. — The MES-resistant and control strains of Pneumococcus type I were first compared for their ability to ferment various substances. No differences between the two strains were observed. They both produced acid and no gas from the following: glucose, lactose, sucrose, trehalose, arabinose, dextrin, and inulin. They both grew out in the presence of the following substances which they failed to ferment: salicin, cellobiose, sorbose, rhamnose, sorbitol, dulcitol, and mannitol.

Rates of growth.—A comparison was next made between the rates of growth of the control and MES-resistant strains during the logarithmic phase of growth only (where the rate is constant). The test was made on 7 tubes for each strain, the tubes containing one common batch of medium; a mean rate of growth measured in terms of bacterial nitrogen was computed and was found to be greater for the MES-resistant strain. A statistical test was made for the significance of the difference between the means, and the *P* value was 0.058. This result is considered suggestive but not statistically significant.

The previous two experiments were carried out in view of the fact that a new strain of microorganism having an increased growth rate and a change in bio-

chemical properties, has been compared by some authors to the new "strain" of tumor cells, which have an increased growth rate and different properties over those of the normal cells from which they were derived.[10,42]

Cross resistance of MES-resistant Pneumococcus type I to DBAS.—The experiments performed with both MES and DBAS on *Lactobacillus* 46 and Pneumococcus type I have indicated the possibility that both carcinogens have a common mode of action. For example, they are both inhibitory in approximately the same concentrations; the action of both is antagonized by autoclaved yeast extract plus phosphate; and they both appear to have a bacteriostatic action in the concentrations studied. To investigate this matter further, it was decided to see whether the MES-resistant pneumococcus strain was also resistant to DBAS. The control strain and the MES-resistant strain of Pneumococcus type I were inoculated into yeast extract mediums containing DBAS in 20 μg/ml concentration. Turbidity readings were taken periodically and bacterial nitrogen determinations were made at the points where the maximum growth of the cultures was reached. The data for the maximum bacterial nitrogen attained by each strain are presented in table 8.

TABLE 8.—*The cross resistance of MES*-resistant Pneumococcus type I to DBAS* in a yeast extract medium.*

	Control strain†	MES-resistant strain†
	DBAS in 20 μg/ml	DBAS in 20 μg/ml
Maximum growth (bacterial nitrogen per 2 ml)	15 μg	55 μg
Increase in resistance to DBAS		3.7-fold

* See table 1.
† Control strain and MES-resistant strain are defined in the text.

The turbidity data (not tabled here) and the bacterial nitrogen data both show that a strain of pneumococcus resistant to MES is also partially resistant to DBAS. Similar results have been obtained with lower concentrations of DBAS. These data in table 8 are presented to support the idea that the inhibitory effects of DBAS and MES may have the same mechanism of action.

The results with the MES-resistant strain are to be regarded as preliminary in nature. Further experiments with the MES-resistant strain were not carried out because it was felt that an investigation with resistant strains would yield more lucrative information if resistance to DBAS (rather than MES) could be developed to a substantial degree and in a defined medium in which the pneumococcus could grow well. An examination of a carcinogen-resistant strain is perhaps one of the most promising approaches towards obtaining further valuable information concerning the mode of action of carcinogens.

DISCUSSION

The investigation undertaken here has shown a definite inhibition, by the two carcinogenic derivatives used, of the growth rates and total growth of certain gram-positive bacteria and only one gram-negative bacterium, *V. cholerae*. The noncarcinogen employed exerted no effect. Not all the gram-positive bacteria tested were inhibited by the carcinogens. In view of the antagonizing action of autoclaved yeast extract plus phosphate against the effect of the carcinogens in the case of *Lactobacillus* 46, it is possible that the use of a growth medium other than the yeast extract medium would uncover more organisms that are susceptible to inhibition by

42. Haddow, A. 1947, Brit. Med. Bull. 4: 331–342.

carcinogenic agents. The inhibition was proportional to the concentration of carcinogen and was first detectable in the case of the stronger DBAS at 0.1 μg/ml (less than 10^{-6} *M*).

Intensive study was continued for *Lactobacillus* 46 and Pneumococcus type I. A study of the environmental influences on the inhibitory power of the carcinogens disclosed that inhibition was most effective at a lower pH within the range studied, that the size of inoculum had no notable influence on inhibition, that younger organisms were more easily inhibited than older ones, and that inhibition was slightly greater under anaerobic conditions. The action of the carcinogens was in some measure differentiated by several tests from that of anionic surface-active agents, which they resemble in general structure and some of whose properties they share. The inability of SH reversing agents to prevent the action of the carcinogens casts doubt on the idea that SH enzymes are a primary site of action. The failure to observe morphological abnormalities such as swollen or filamentous forms indicated that the action was different from compounds such as penicillin.

Further studies indicated that the inhibition by the carcinogens was probably bacteriostatic in character, which probably involves the assimilation and/or the synthetic mechanism. This tentative conclusion is based on the inability to find a killing effect of the carcinogens in survival experiments and the failure to detect an effect of the carcinogens on the aerobic respiration of Pneumococcus type I (glucose as substrate) or on the anaerobic dissimilation of glucose. In addition, Pneumococcus type I and *Lactobacillus* 46 showed the ability to grow out upon dilution, and, in the case of *Lactobacillus* 46, upon the addition of a reversing agent(s), even after 48 hours of contact with the carcinogen in the incubator. These combined results show an action that is in contrast to most "nonspecific" cell poisons. The failure to demonstrate an action by the hydrocarbon derivatives on the carbohydrate metabolism or respiration of Pneumococcus type I, even after special efforts were made, is in contrast to results reported on yeasts[10,22,23] for various carcinogens. It is also in contrast to the results reported on animal tissue enzymes for the split products of azo dye carcinogens and to certain work with hydrocarbons most of which implicate the sulfhydryl enzymes or the sulfur metabolism of higher organisms.[43,44] However, the "negative" results with enzyme systems in this work are in agreement with some published experiments on enzyme systems[10,14,32] and with many unpublished experiments of failures to show effects of carcinogens on enzyme systems in animal tissues. The differences in results may be due in part to the different experimental conditions, or to the inherent difficulty with any problem that may involve the synthetic reactions of organisms, of which little is known. Whatever may be the mode of action of the hydrocarbon derivatives on bacteria, it would appear that DBAS and MES act similarly. This is based on the fact that autoclaved yeast extract plus phosphate solutions were able to reverse the inhibitory effects of both carcinogens on *Lactobacillus* 46, and an MES-resistant strain was found to possess cross resistance to DBAS. Moreover, wherever both carcinogens were tested, they produced the same effects in approximately similar concentration, even though it is recognized that the

43. Crabtree, H. G. 1948, Brit. J. Cancer, **2**: 281–289.
44. Kensler, C. J. 1947, Ann. N. Y. Acad. Sc. **49**: 29–40.

effects tested were broad in nature.

One of the most interesting facts disclosed in this investigation was the ability of certain lots of yeast extract autoclaved together with phosphate to prevent and reverse the action of the two carcinogens on *Lactobacillus* 46 in the semisynthetic medium. The fact that only combinations autoclaved together with phosphate were active as reversing agents is not strange in the light of the fact that the active forms of many essential nutrilites are phosphate combinations (see Snell[45]). The antagonistic and reversing action of the autoclaved yeast extract plus phosphate on the growth inhibition of *Lactobacillus* 46 suggests experiments which may aid in determining whether the inhibition by the carcinogens is of the competitive type.[38] Exact quantitative experiments to test this have not yet been carried out.

In contrast to the results with *Lactobacillus* 46, it is important to note that the yeast extract medium could not prevent the inhibitory action of the carcinogens on the pneumococcus. This may be due to a quantitative difference in the need for the reversing agent(s), or to the fact that a different form of the agent(s)—if it is a vitamin, for example—is utilized by the pneumococcus. It is also possible, of course, that the mode of action of the carcinogens is different for the two organisms.

There is a whole field within cancer research devoted to the influence of nutrition in the prophylaxis and treatment of cancer.[29,46] Several favorable reports attesting to the effectiveness of certain nutrilites have appeared, som of which are confined strictly to the tumors caused by specific chemicals.[10,44,47] On the basis of experiments which showed that boiled yeast could antagonize the inhibitory action of the carcinogen styryl 430 on a certain strain of yeast, the boiled yeast was used successfully as a prophylactic agent against benzpyrene-induced cancer in mice.[10] Riboflavin plus casein were found to be effective in preventing tumors induced by azo dye carcinogens.[44] Subsequently, it was found that *L. casei* could be inhibited by an azo dye carcinogen and that high levels of riboflavin could, in part, prevent the inhibition.[9] In view of these facts, the discovery that the inhibition of the growth of *Lactobacillus* 46 by carcinogens can be prevented and reversed by autoclaved yeast extract plus phosphate may possibly have significance. It is important to keep in mind, however, that it is a long way from the antagonism of the inhibition of the growth of *Lactobacillus* 46 by carcinogens to prevention of tumors in animals.

Attention should also be given to the preliminary experiments recorded for the MES-resistant strain. An investigation of differences in nutritive requirements, in the immunological specificity of various bacterial fractions, and in other physiological properties, between a bacterium made highly resistant to a carcinogen and its susceptible counterpart may yield valuable information concerning the comparative autonomy of the two strains, and the mode of action of the carcinogens.

SUMMARY

The action of three water-soluble hydrocarbon derivatives, sodium methylcholanthrene endosuccinate (MES), 1,2,5,6-dibenzanthracene endosuccinate (DBAS), and sodium anthracene endo-

45. Snell, E. E. 1942, Proc. Soc. Exper. Biol. & Med. 51: 356–358.
46. Nutrition in Relation to Cancer, 1947, Ann. N. Y. Acad. Sc. 49: 1–140.
47. Lewisohn, R. L., Leuchtenberger, C., Leuchtenberger, R., Lazlo, D. and Bloch, K. 1941, Cancer Research, 1: 799–806.

succinate (AES), on bacterial growth and metabolism was investigated. It was found that the two carcinogenic derivatives (MES and DBAS) inhibited and never stimulated the growth of various gram-positive bacteria and one gram-negative bacterium. The non-carcinogen AES exerted no effect. The inhibition of growth by the carcinogens was approximately proportional to the concentration of the carcinogen.

Further intensive studies using *Lactobacillus* 46 and Pneumococcus type I were made which included respiration experiments, survival-type experiments, a study of the influence of various environmental factors on the inhibitory action of the carcinogens, etc. On the basis of the evidence accumulated for the two organisms, it is concluded that the carcinogens DBAS and MES appear to exert a similar bacteriostatic action on bacteria, in which the assimilation and/or synthetic reactions are probably involved. The action of the carcinogens was discussed in relation to other bacterial inhibitors and in relation to the present evidence regarding the action of chemical carcinogens.

In addition, a factor(s) was found in certain autoclaved yeast extract plus phosphate solutions which could prevent and reverse the inhibitory action of the carcinogens on *Lactobacillus* 46.

STUDIES IN THE PATHOLOGY AND PATHOGENESIS OF EXPERIMENTAL BRUCELLOSIS

I. A COMPARISON OF THE PATHOGENICITY OF *BRUCELLA ABORTUS*, *BRUCELLA MELITENSIS*, AND *BRUCELLA SUIS* FOR GUINEA PIGS

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The effects of inoculating guinea pigs with brucella from different sources have been compared mainly by Meyer, Shaw, and Fleischner,[1] Cotton,[2] and Smith.[3] Meyer et al[1] and Cotton[2] were the first to emphasize that the widespread suppuration produced in infected guinea pigs by brucella from porcine sources far exceeded that observed after the inoculation of brucella isolated from cows. Smith[3] compared the disease in guinea pigs produced by two strains of *Brucella melitensis* with that produced by two strains of *Brucella abortus* and found that in the group infected by *Br. melitensis* there was less gain in weight and larger focal necrotic lesions in the liver than in animals infected with *Br. abortus*.

These studies were all published from 1922 to 1926 and before Huddleson[4] clearly separated the three species of brucella in 1929. The identity of the organisms used in these earlier experiments must, therefore, remain uncertain particularly in the case of *Brucella suis* and *Br. abortus*, as both species were included in the category of "*Br. abortus*." There has been a need for a reconsideration of the relative pathogenicity of various strains of brucella from the standpoint of Huddleson's classification. The present study was conducted to provide this information chiefly with respect to brucella possessing the common property of known pathogenicity for man.

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1. Meyer, K. F., Shaw, E. B. and Fleischner, M. C. 1922, J. Infect. Dis. **31**: 159–197.
2. Cotton, W. E. 1922, J. Am. Vet. M. A. **15**: 179–192.
3. Smith, T. 1926, J. Exper. Med. **43**: 207–223.
4. Huddleson, I. F. 1931, Am. J. Pub. Health, **21**: 491–498.

MATERIALS AND METHODS

Smooth strains of brucella from 18 sources were selected at random; there were 6 strains of *Br. abortus*, 6 of *Br. suis*, and 6 of *Br. melitensis*. All were isolated from patients in Minnesota or Mexico City with the exception of one strain of *Br. suis* and one strain of *Br. melitensis* obtained from the National Institute of Health, Bethesda, Maryland. The Mexican strains were supplied by Dr. M. R. Castaneda. A total of 54 male guinea pigs obtained from the same source were each inoculated intraperitoneally simultaneously with approximately 500,000 cells from a young culture. Each of the 18 strains was injected into three animals. The inoculums were prepared from cultures grown for 24 hours on tryptose agar and suspended in 0.5 ml of physiologic saline solution. The concentration of bacteria was determined by adjusting the density of the suspension in a photoelectric colorimeter. A standardized colorimeter reading was obtained with a suspension of 1 billion brucella cells, the number of which was determined by counting the colonies in an inoculated pour plate.

Animals were permitted to survive for 130 days after inoculation. This interval of time was calculated on the basis of preliminary experiments

which disclosed that a period of 3 to 6 months was required for lesions to reach full development in guinea pigs infected by any of the three species of brucella. It was also learned in the preliminary studies that numerous deaths occurred within 80 days among small (250 to 350 g) guinea pigs infected by *Br. suis* or *Br. melitensis*. To prolong the survival period, the present group of 54 guinea pigs was composed of older and heavier animals weighing about 500 g.

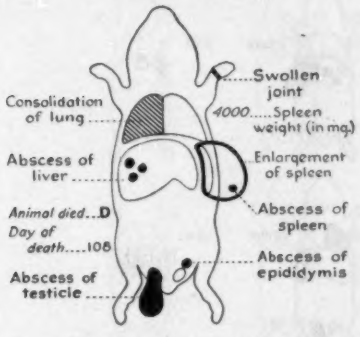


FIG. 1.—Diagram of guinea pig used for illustrating gross pathologic changes in experimental brucellosis. (Modified from Feldman and Hinshaw.)[6]

Observations were made with regard to general appearance and change in weight during the course of the infection. At the time of sacrifice, blood was obtained by cardiac puncture for culture and agglutination tests. Blood cultures were performed by the injection of 3.0 to 5.0 ml of blood directly through the rubber stopper of a sealed bottle containing the double medium described by Castaneda.[5] For growth of *Br. abortus*, carbon dioxide in a concentration of 10% was added to the individual bottles. Subcultures were made from the broth or from colonies on the slant at regular intervals up to one month at which time the cultures were discarded. Post mortem examination of each animal was performed immediately after its death. Prior to dissection of the sacrificed guinea pigs, they were immersed in a solution of 5% cresol to flatten the fur and to reduce contamination. Cultures of organs were made streaking the cut sections on tryptose agar.

Agglutination tests were performed on blood serums by the standard tube method. An antigen was used which is produced by the Bureau of Animal Industry, Bethesda, Maryland. The mixtures of antigen and serum were incubated in the water bath at 37 C for 48 hours, and read immediately afterwards without chilling.

Careful records were made of the pathologic changes observed at autopsy. The schematic representations, which were employed for illustrating the gross lesions in figures 1 to 4, were modelled after those originated by Feldman and Hinshaw[6] for recording lesions of experimentally induced tuberculosis of guinea pigs.

For histologic study, blocks of tissues were fixed in a solution of 10% formalin in water or saline, and stained by the hematoxylin and eosin stain by Giemsa's method, and by the azocarmine method.

RESULTS

Comparison of the general effects of infection by Br. abortus, Br. suis *and* Br. melitensis

Infection by Br. suis.—Only one guinea pig died before the termination of the experiment. The survivors appeared in a fairly good state of health. The average gain in weight was 126 g. There were larger and more numerous abscesses among the animals infected by *Br. suis*, and those lesions involved the greatest number of animals of any of the three groups. Every strain of *Br. suis* caused the development of abscesses which could be demonstrated macroscopically in all but two guinea pigs (fig. 2).

Infection by Br. melitensis.—There were three spontaneous deaths. More animals with outward signs of poor health were found among those infected with *Br. melitensis* than in the other two groups. The signs included roughening of the fur, loss of hair, listlessness, and an average gain in weight of only 119 g. Only three strains of *Br. melitensis* caused the development of grossly visible abscesses, and these were present in a total of 5 animals (fig. 3). Microscopic lesions were noted in the tissues of 9 guinea pigs (50%).

5. Castaneda, M. R. 1947, Proc. Soc. Exper. Biol. & Med. **64**: 114–115.

6. Feldman, W. H. and Hinshaw, H. C. 1945, Am. Rev. Tuberc. 582–591.

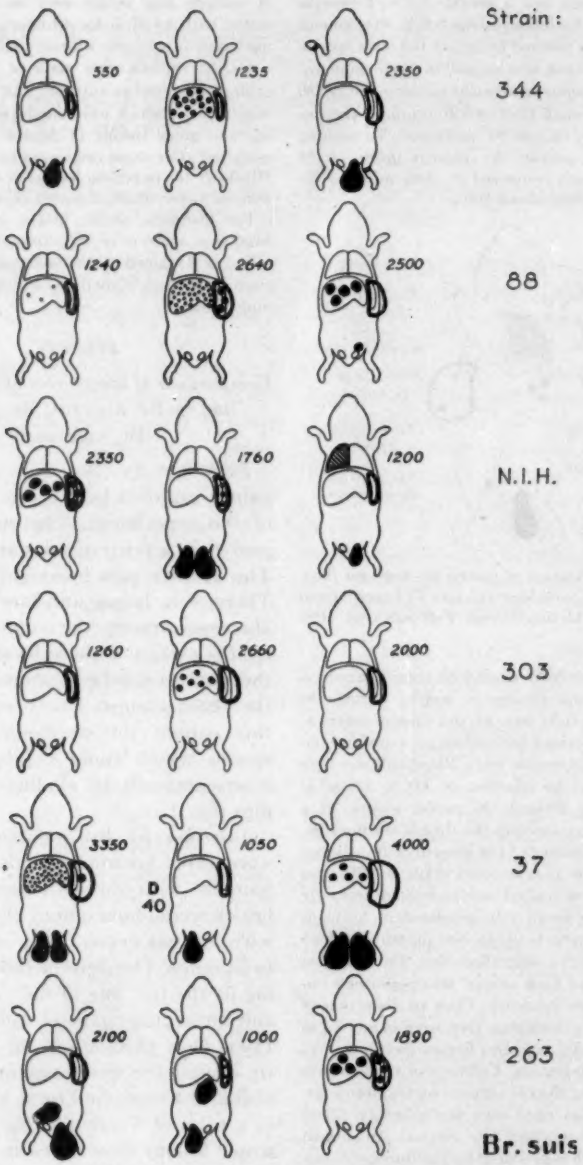


FIG. 2.—Diagram of gross abnormalities observed in guinea pigs approximately 130 days after infection by one of 6 different strains of *Br. suis*.

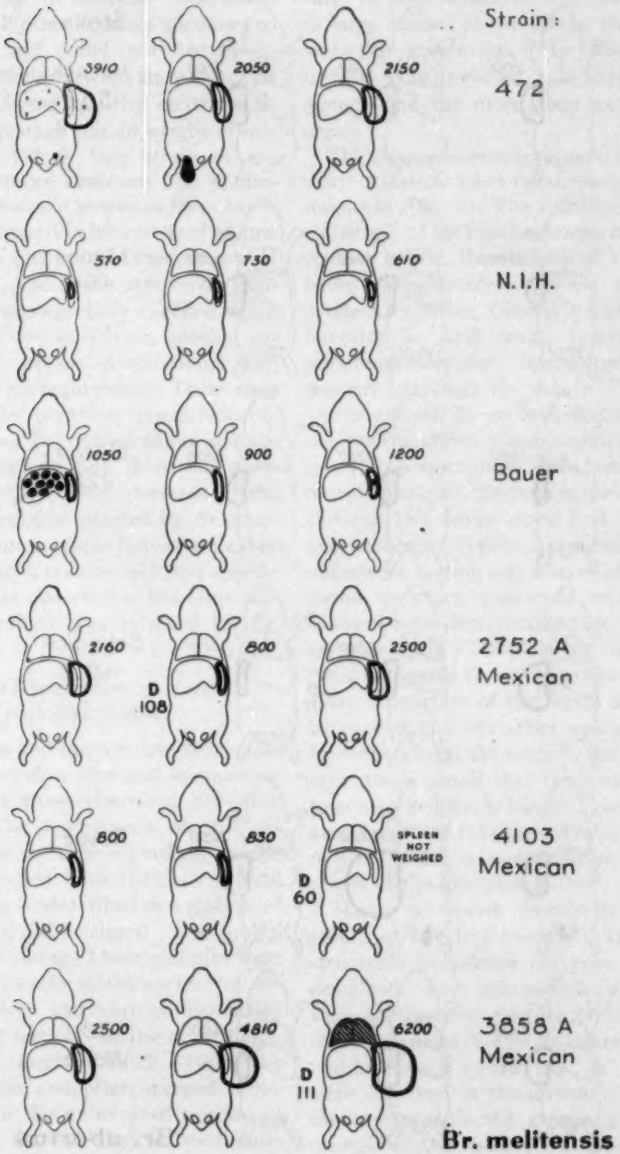


FIG. 3.—Diagram of gross abnormalities observed in guinea pigs approximately 130 days after infection by one of 6 different strains of *Br. melitensis*.

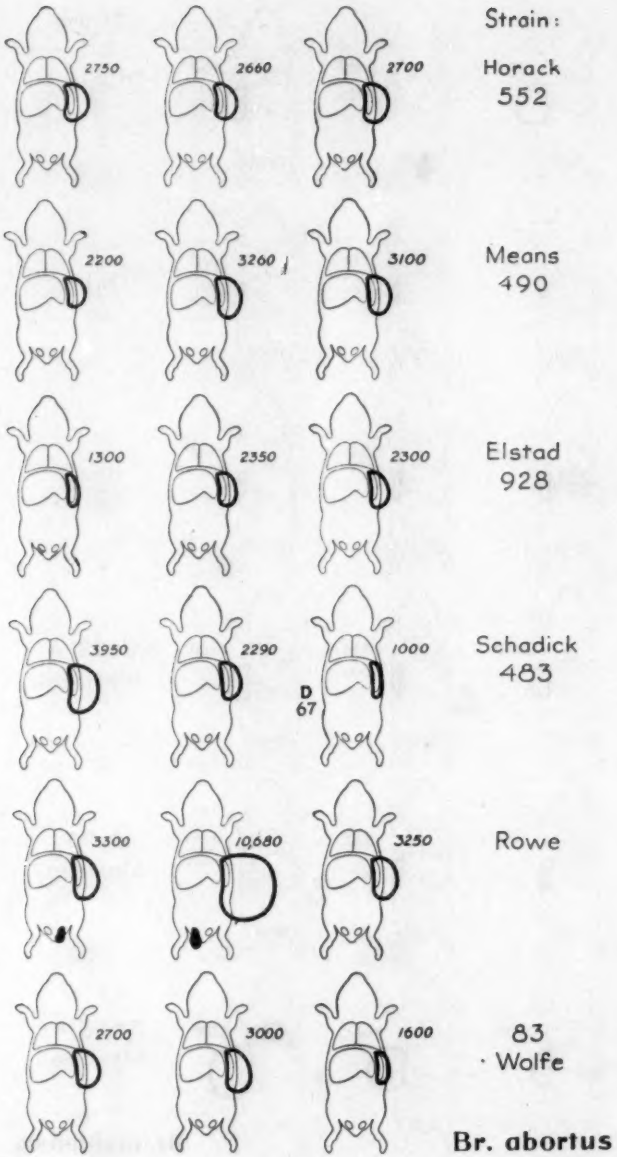


FIG. 4.—Diagram of gross abnormalities observed in guinea pigs approximately 130 days after infection by one of 6 different strains of *Br. abortus*.

Infection by Br. abortus.—One death occurred. All other animals appeared to be robust and could not be distinguished by their outward state of health from noninfected healthy control animals. The average gain in weight in this group was 180 g. One strain of *Br. abortus* produced abscesses (fig. 4). Because macroscopic abscesses have never been encountered in infections of guinea pigs due to any of 13 other strains of *Br. abortus*, the strain recovered from this lesion was carefully checked again by tests of dye inhibition, specific agglutination, urease production,* and carbon dioxide requirement. These tests classified the organism unequivocably as *Br. abortus*. In contrast to the scarcity of macroscopic lesions, there was widespread microscopic involvement of the tissues of animals infected by *Br. abortus*. When microscopic lesions are taken into account, it may be said that specific changes were observed in the large majority of guinea pigs infected by *Br. abortus*.

Detailed description of specific pathologic lesions

The gross and microscopic characters of the lesions were identical in many respects with those described by other authors. The appearance of the abscesses was uniform regardless of the species cultured from them. A typical abscess may be described as a globule of cheesy material enclosed in a tough, thin, white casing. These globules were embedded loosely in the surface of the liver or spleen and were easily shelled out. In the testicle, on the other hand, they were firmly bound within the fibrous tunic and often merged indistinctly with the uninvolved portion of the gland. Rarely, they existed independently of any recognizable structure. In such situations they were seen as large masses in the pelvis, the neck, and the mesentery. The liver and testicle were involved with equal frequency and far more than any other organ.

The abscesses were composed of three distinct parts when examined microscopically (fig. 5). The center, or core, consisted of a homogeneous mass of cellular debris, the outlines of the cells being recognizable in some sections stained by either Giemsa's method or hematoxylin and eosin. Innumerable polymorphonuclear leucocytes were scattered through the debris. The core was enveloped by an intermediate zone of granulomatous tissue composed primarily of epithelioid cells and polymorphonuclears. Sections made directly through this layer alone had the appearance of a typical granuloma. The outermost portion was a layer of fibrous tissue in which numerous strands of collagen were demonstrated by the azocarmine stain. These fibers formed a circular capsule about the entire abscess. That proportion of the lesion occupied by each of the two zones was variable. In some places, the necrotic center was exceedingly small and the granulomatous area relatively large. This appearance suggested that the necrotic portion resulted from a degeneration of the center of the granuloma.

Many abscesses remained microscopic in size but possessed the same structural properties of those already described. The microscopic abscesses were encountered frequently except in those infections due to *Br. abortus*. Only two abscesses caused by *Br. abortus* were observed in innumerable sections made from infected guinea pigs and mice. For the most part, granulomas produced by any one of the strains of *Br. abortus* did not develop into abscesses, and fibrous capsules were ab-

* The test for urease production was performed by Dr. Henry Bauer.

TABLE 1.—*Frequency of involvement of various organs by macroscopic or microscopic lesions in 54 guinea pigs inoculated intraperitoneally with one of 18 strains of* Brucella.

Organ	Total number of guinea pigs in which lesions were noted
Liver	31
Testicle and epididymis	17
Kidney	11
Bones (not including bone marrow)	2
Lungs	2

sent. Aside from the collagen demonstrated in the wall of the abscesses, the inflammatory process was not associated with fibrous tissue formation.

The appearance of the microscopic changes was determined in general by the organ in which they were located. Table 1 lists the order of frequency with which various organs were involved by either macroscopic or microscopic lesions, without respect to the species of brucella. The spleen is not included in the tabulation because it was usually difficult to distinguish nonspecific cellular reactions from those which were believed due specifically to the invasion of that organ by brucella. Lymph nodes are also omitted from the table, as these organs were not consistently found at autopsy.

Evidence of disease was apparent most often in the liver, an organ containing an abundance of reticulo-endothelial elements. In 60% of all animals, well circumscribed granulomas or abscesses were present which were located in any portion of the lobule and sometimes occupied an area equivalent in size to several lobules. Periportal hepatitis was often produced by a heavy diffuse infiltration of epithelioid cells as well as variable numbers of polymorphonuclear leucocytes, lymphocytes, and plasma cells. As illustrated in figure 6, this process in the periportal areas occasionally became so extensive that the appearance resembled cirrhosis of the liver. When special stains for connective tissue were applied, however, a significant increase in collagen in these areas was never demonstrated. Focal granulomas and abscesses were also encountered in the epididymis and testicle, the two types sometimes occurring simultaneously. Microscopic evidence of disease of the kidney was present in about 20% of the infected animals, but there were no gross abnormalities. The renal changes differed from those described in the liver and testicle. Instead of being sharply circumscribed, the lesions extended irregularly and diffusely through the interstitial portion of the cortices. This process was similar to that in the periportal areas of the liver and contained the same cellular elements. As shown in figure 7, the infiltration sometimes extended to the outer layer of Bowman's capsule but did not appear to invade the glomerulus. Necrosis, suppuration, or fibrosis did not develop, and no special features peculiar to any of the three species of infecting organisms was found.

Pulmonary lesions were found in only two animals, one of them infected with *Br. suis* and one with *Br. melitensis*. In both cases, there was an extensive interstitial pneumonia which had spread throughout an entire lung. The organs became heavy and airless, sank in water, and offered resistance to section. The underlying inflammatory reaction was granulomatous, but in the animal infected by *Br. suis*, there was also marked suppuration and fibrosis.

The osseous deformities were typical of those described by others and will not be discussed here. They were not common, occurring in only two animals, both of which were infected by *Br. suis*. In another group of 60 guinea pigs inoculated with *Br. suis* (strain "37"), only 4 animals developed visible lesions of bone after a period of observation

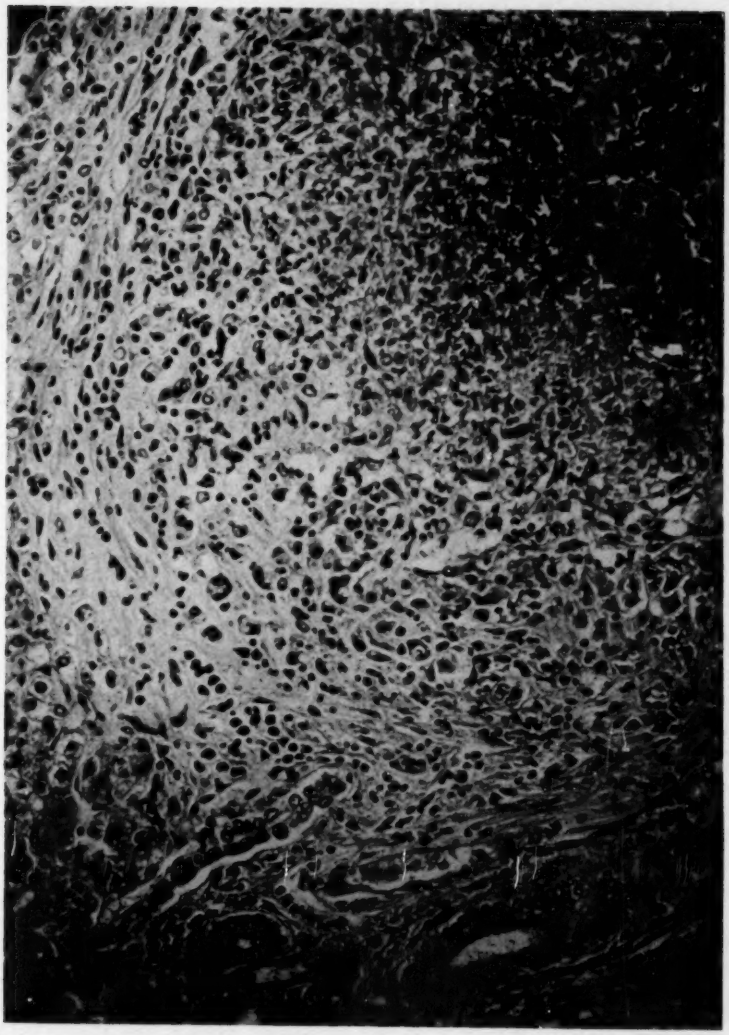
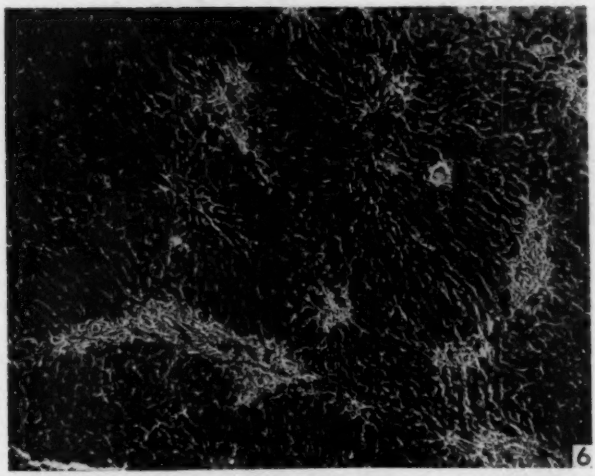


FIG. 5.—Section through abscess found in liver of guinea pig infected by *Br. suis*. Note inner zone of necrosis, intermediate zone of granulomatous inflammation, and outer fibrous capsule. (Hematoxylin and eosin stain, ×250.)



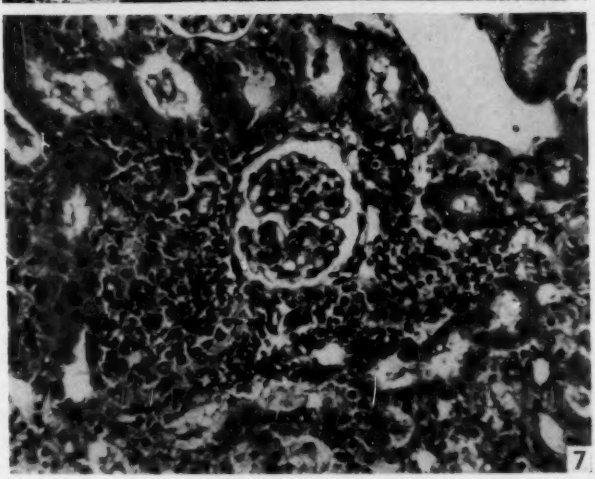


FIG. 6.—Portal hepatitis in liver of guinea pig infected by *Br. melitensis*. (Hematoxylin and eosin stain, ×100.)

FIG. 7.—Interstitial inflammation of granulomatous type in kidney of guinea pig infected by *Br. suis*. The process extends up to Bowman's capsule but does not invade it. (Hematoxylin and eosin stain, ×250.)

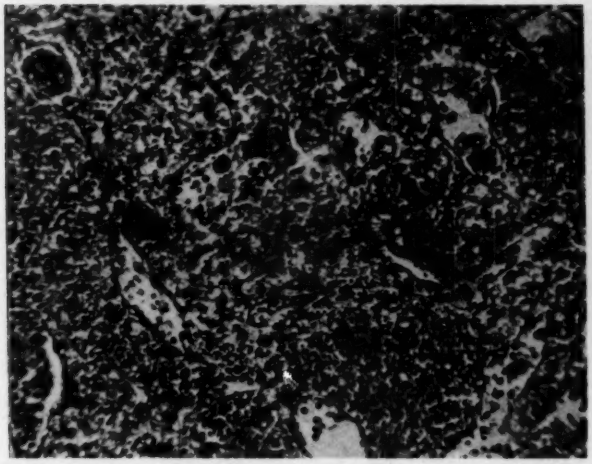


FIG. 8.—Spleen from guinea pig infected with *Br. melitensis*. Spleen weighed 5 g, and *Br. melitensis* was cultured from it at autopsy. Azocarmine stain shows no increase in fibrosis. Note congestion of splenic sinuses and pulp spaces. (Azocarmine stain, ×75).

similar to that of the present experiment. Experience with hundreds of infected guinea pigs indicates that disease of bone is even much more unusual in the case of experimental brucellosis due to *Br. abortus*. Granulomas in microscopic sections of the bone marrow, on the other hand, are often present.

The weight of every spleen removed from animals infected by *Br. abortus* was greater than 1.0 g. The heaviest spleen encountered among normal guinea pigs of the same age and weight was 850 mg. Splenic enlargement was nearly as constant in the group infected with *Br. suis*, as this feature was absent in only a single guinea pig. Only two-thirds of the spleens from guinea pigs infected with *Br. melitensis* were heavier than 850 mg.* The chief cause of enlargement in every case was engorgement of the splenic sinuses and pulp spaces. Hyperplasia of the cellular elements also occurred but this was difficult to assess both from the standpoint of degree as well as quality. It was not possible to distinguish accurately between spleens of infected animals and those of normal animals on the basis of microscopic study alone. Fibrosis was absent and did not contribute to the size of the spleens. Figure 8 is a photograph of the section of a spleen which weighed nearly 5 g and from which *Br. melitensis* was recovered 18 weeks after inoculation.† No increase in fibrous tissue can be seen after staining with azocarmine.

DISCUSSION

A general summary is presented in table 2. *Br. suis* produced the most numerous and destructive lesions, and the general condition of the infected animals was fair. Animals infected by *Br. abortus* rarely displayed more than

* Actually, it does not appear to be necessary to make corrections for the weight of the animal in comparing the weight of spleens from guinea pigs of different size. There is very little difference in the weight of spleens among guinea pigs ranging in size from 300 to 900 g (Webster, S. H. and Liljegren, E. J. 1949, Am. J. Anat. **85**: 199–230.)

† This spleen belonged to guinea pig (B) inoculated with Strain 385A (Mexican).

TABLE 2.—*A comparison of the effects produced by the inoculation of guinea pigs with various strains of* Br. abortus, Br. suis, *and* Br. melitensis.

Species of *Brucella*	Total Number of different strains used	Total number of guinea pigs infected by each species	Total number of strains producing abscesses by gross examination	Total number of guinea pigs in which abscesses were produced	Average weight in g gained by guinea pigs infected by each species	Average weight in mg of spleens of guinea pigs infected by each species
Br. abortus	6	18	1	2	180	2996
Br. suis	6	18	6	16	126	2043
Br. melitensis	6	18	3	5	119	1932

microscopic evidence of involvement of tissues with the development of nonsuppurative granulomas. They withstood the infection with little sign of impaired health, and the course of the disease was characterized by marked enlargement of the spleen and excellent gain in weight. *Br. melitensis* appeared to be the most virulent of the three species, if attention was directed to the poor health of the infected guinea pigs and the poor average gain in weight. In spite of this, gross pathologic changes were far less in these animals than in those inoculated with *Br. suis*, and even the microscopic changes were less numerous than in the case of infections by *Br. abortus*. It appears significant, also, that *Br. melitensis* did not cause consistent enlargement of the spleen.

From these findings, it appears that the good defense of guinea pigs against *Br. abortus* was associated with the presence of the nonsuppurative granuloma and marked splenomegaly. The less effective defense against *Br. suis* was associated with the development of widespread suppuration. *Br. melitensis*, intermediate in its destructive capacity, was the most debilitating. This information indicates that another factor, possibly an endotoxin, plays a part in the evolution of the experimental disease.

SUMMARY AND CONCLUSIONS

1. A comparison has been made of the pathogenicity of the three species of brucella for guinea pigs.

2. In guinea pigs inoculated with *Brucella suis* there was formation of abscesses in almost every animal. The tendency for formation of abscesses was much less striking after inoculation of *Brucella melitensis*, but the animals manifested a more severe illness than those infected with *Br. suis*. It was found that *Brucella abortus* produced only slight destruction of tissues.

STUDIES IN THE PATHOLOGY AND PATHOGENESIS OF EXPERIMENTAL BRUCELLOSIS

II. THE FORMATION OF THE HEPATIC GRANULOMA AND ITS EVOLUTION

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The granuloma is the basic histologic lesion in the tissues of animals and man after infection with brucella. This was emphasized first in 1912 by Fabyan,[1] whose observations were carried out in guinea pigs. In subsequent years, the occurrence of the granuloma of brucellosis has been reported in the tissues of mice by Feldman and Olson,[2] of monkeys by Huddleson and Hallman,[3] of dogs by Margolis et al,[4] and of rats and rabbits by Nyka.[5] Spink et al[6] have observed granulomas in sections of liver obtained by biopsy from many patients having culturally proven brucellosis. Similar findings in sections of biopsied human bone marrow were reported by Sundberg and Spink.[7]

Although the uniform character of the cellular reaction to brucella in various hosts is well established by these reports, the significance of the granuloma is poorly understood. The sequence of events involved in the development of the characteristic focal lesions has been the subject of speculation, but the formation and evolution of the granuloma has never been established experimentally. The close morphologic resemblance between the granulomas of brucellosis and tuberculosis suggests that both diseases may have a similar pattern of tissue reaction.

The consistent occurrence of hypersensitivity is another similarity between brucellosis and tuberculosis, and one which could possibly have a bearing on the histopathologic changes in both diseases. However, Follis,[8] Rich and McCordock,[9] Vorwald,[10] and Rothschild et al[11] have presented evidence that hypersensitivity is not essential to the development of the tubercle. Follis[8] and Rothschild et al[11] demonstrated tubercle formation in tuberculous animals in which hypersensitivity was prevented by repeated injections of tuberculin. After the introduction of tubercle bacilli into the normal rabbit, Vorwald[10] found evidence of tubercle formation 24 hours later, a period which is probably

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too short for hypersensitivity to develop. There are no reports of studies pertaining to the association of hypersensitivity and the granuloma in brucellosis.

The fate of the granuloma in brucellosis has also been an unsolved problem. It has been demonstrated in experimental tuberculosis, that the tubercle may become a nodule of fibrous tissue or may undergo complete resolution and disappear after dispersal of the component cells.[11]

In a study reported elsewhere, it was found that the nonsuppurative granuloma was associated with a good defense of guinea pigs against *Brucella abortus*, while the suppurative granuloma was found in animals exhibiting poorer resistance to *Brucella suis* or to *Brucella melitensis*.[12] It was also noted after intraperitoneal inoculation that granulomas were present more frequently in the liver than in any other organ. In an effort to learn more about the significance of the granuloma, the development of the nonsuppurative granuloma was traced from the earliest stages of formation to the final outcome by serial examinations of mice and guinea pigs. Interest was focused on the changes in the liver because the effect upon both parenchymal and reticuloendothelial cells may be studied here simultaneously, and because specific lesions occur more frequently in this organ than in any other. Experiments were also conducted to study the possible relationship between the development of the granuloma and the presence of hypersensitivity in experimental brucellosis.

MATERIALS AND METHODS

Guinea pigs and mice were infected with varying doses of brucella by intracardiac or intra-abdominal puncture and sacrificed at different intervals after inoculation. Tissues were examined for the purpose of observing the basic cellular response in various stages. It was observed that the character of the specific lesions did not vary with the size of the inoculum, although the number of lesions was greatly increased by the use of a massive number of organisms. One small dose of aureomycin was found to protect mice receiving lethal injections of 100 billion organisms, but the character of the resulting tissue changes did not differ from those of untreated animals receiving sublethal inoculums.[13]

White Swiss mice weighing 20 g were used for studying the acute reactions in the tissues. These animals received intraperitoneally 100 billion actively growing cells of the "Lynch" strain of *Br. abortus* and were simultaneously given 0.4 mg of aureomycin hydrochloride subcutaneously. Smears of blood from the tail were studied at 15 minute intervals until brucella organisms were observed in the blood, and then hourly thereafter for 6 hours. Mice were sacrificed at periods of 3, 6, and 24 hours after inoculation, and then daily until the 6th day.

Brucella organisms were easily found in the blood and in the tissues of mice during the first few days of the infection. Various stains were employed, including those of Wright, Giemsa, and Nyka, as well as ordinary methylene blue. Giemsa's stain or methylene blue proved satisfactory for demonstrating the organisms in blood smears and tissue imprints, and a modification of Giemsa's method gave best results for staining brucella in the tissues.

The development of the more chronic lesions was traced in guinea pigs. The size of the inoculum was chosen with the purpose in mind of establishing a chronic but sublethal infection to be observed over a period of one year. Guinea pigs weighing about 350 g were infected by intracardiac inoculation with 500,000 cells of the "Lynch" strain of *Br. abortus* or 5 billion cells of strain "19."* These animals were sacrificed after periods of infection of 5 days, 8 days, 10 days, 1 month, 3 months, 6 months, and 1 year.

For rendering guinea pigs anergic, a group of 24 were given weekly subcutaneous injections of increasing doses of heat-killed *Br. abortus*. Each animal had been infected by intracardiac inoculation with either 500,000 cells of the virulent "Lynch" strain of *Br. abortus* or 5 billion cells of strain "19." Homologous strains were used for

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*Strain "19" is a culture of *Br. abortus* of low virulence provided by the Bureau of Animal Industry for vaccination of cattle to control brucellosis.

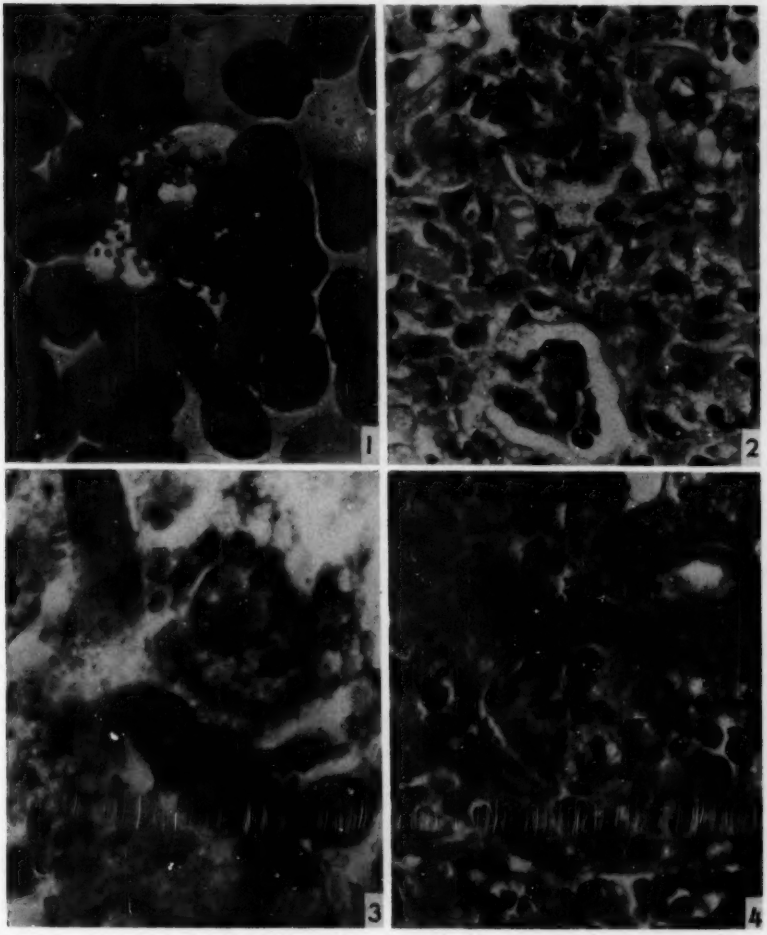

FIG. 1.—Phagocytosis of *Br. abortus* by polymorphonuclear leucocyte in blood of mouse 3 hours after intraperitoneal inoculation of 100 billion *Br. abortus*.

FIG. 2.—Focal aggregates of parasitized polymorphonuclear leucocytes collecting in sinusoids of liver of mouse sacrificed 6 hours after intra-peritoneal inoculation of 100 billion *Br. abortus* (Giemsa stain, ×1700).

FIG. 3.—Liver of mouse 24 hours after intraperitoneal inoculation of 100 billion *Br. abortus*. Note Kupffer's cells distended by brucella and filling sinusoid. (Giemsa stain, ×4000).

FIG. 4.—Liver of mouse 120 hours after intraperitoneal inoculation of 100 billion *Br. abortus*. Granuloma is present, composed almost exclusively of epithelioid cells. (Giemsa stain, ×1000).

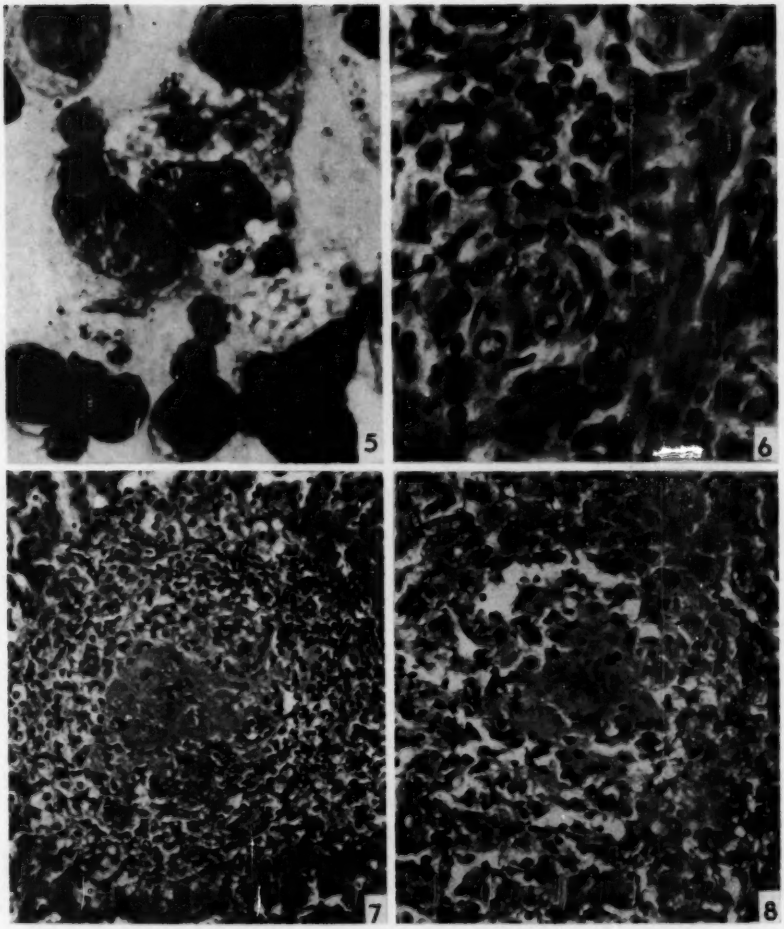

FIG. 5.—Clusters of *Br. abortus* in cytoplasm of histiocyte in imprint made from spleen of mouse sacrificed 72 hours after intraperitoneal inoculation.

FIG. 6.—Liver of guinea pig one month after inoculation with *Br. abortus*. Large focal lesion is composed of numerous smaller granulomas. High power view to show fusion of small granulomas (Hematoxylin and eosin, ×900).

FIG. 7.—Liver of guinea pig 3 months after inoculation of *Br. abortus*. A clear area resembling hyaline necrosis has appeared in center of the granuloma. (Hematoxylin and eosin, ×250).

FIG. 8.—Liver of guinea pig 6 months after inoculation of *Br. abortus*. There is a reduction in the number of cells surrounding the hyaline center in the granuloma. Compare with figure 7. (Hematoxylin and eosin, ×300).

preparation of the heat-killed suspension of organisms, and the initial subcutaneous dose of heat-killed cells was equal to one-tenth of the dose of the living organisms used for establishing the infection. The initial desensitizing injection was made when the infecting organisms were inoculated. Injections of increasing numbers of dead brucella were repeated at weekly intervals until maximum doses of 33 billion organisms of strain "19" and 2.4 billion organisms of the "Lynch" strain were reached.* Animals were considered anergic at the time of sacrifice if no visible intradermal reaction resulted from the injection of 0.3 ml of both brucellergen and a suspension of 20 million heat-killed homologous organisms and if no systemic reactions were detected after the weekly subcutaneous injections of vaccine. The majority of animals could not be desensitized and many died from the large subcutaneous inoculums of killed organisms. Those guinea pigs in which desensitization was accomplished were sacrificed with untreated infected control animals at intervals of 1, 3, and 6 months. Their organs were cultured for brucella and sections made for microscopic study. Blood from the heart was also cultured and tested for agglutinins.

RESULTS

Evolution of the granuloma during the first 5 days of infection in mice

Brucella first appeared in smears of mouse blood about 90 minutes after inoculation. At this time, as many as 6 to 8 organisms were found in a few polymorphonuclear leucocytes. A variable number of extracellular organisms were also seen scattered throughout the smear. The tendency for brucella to be concentrated in polymorphonuclear leucocytes became more apparent in smears taken a few hours later (fig. 1). After 24 hours, extracellular brucella were rarely, if ever, observed in smears of the peripheral blood but were readily found in the circulating polymorphonuclear leucocytes, as long as 5 days after inoculation. They were also seen in large mononuclear cells circulating in the blood at this time.

In sections of the livers of mice sacrificed after 3 hours, brucella were seen in the polymorphonuclear leucocytes which circulated in increased numbers in the sinusoids. Brucella were also observed in the Kupffer's cells. At 6 hours, a remarkable picture was noted. Every sinusoid was filled with many cells containing brucella. Both polymorphonuclear leucocytes and Kupffer's cells were heavily involved. In numerous places within the sinusoids, parasitized polymorphonuclear leucocytes were collected into a focal aggregation in which one or more Kupffer's cells containing brucella were sometimes included (fig. 2). At 24 hours, the most striking feature in the sinusoids was a reduction of circulating polymorphonuclear cells and an increase in number of brucella within Kupffer's cells. The brucella within the cytoplasm of Kupffer's cells were so numerous that they produced a swelling of the cells which appeared to obliterate the sinusoids (fig. 3). At this period of observation and as late as 72 hours, Kupffer's cells lying adjacent to each other were found in small groups similar to collections of polymorphonuclear leucocytes seen at earlier stages. By 120 hours, the cells now formed large aggregates which had the typical appearance of granulomas. Two types of granulomas were distinguished. One of these was composed almost entirely of epithelioid cells, and the other of a mixture of epithelioid cells and polymorphonuclear leucocytes (fig. 4). Brucella were demonstrated with difficulty in these lesions.

The intracellular location of brucella was also observed in other organs. The cells of the spleen were heavily invaded by brucella and imprints of this organ were very satisfactory for studying the distribution of brucella within the histiocytes. There was a constant tendency for the organisms to form clusters in separate parts of the cells. This ar-

* The weekly increment was equal to 50% of the preceding dose of dead brucella.

rangement of the brucella suggested that they were confined to compartments within the cytoplasm (fig. 5).

Development of the granuloma in guinea pigs after the 5th day of infection

In guinea pigs infected with sublethal doses of *Br. abortus*, granulomas present in sections of the liver at 5 days were the same as those seen in the mice sacrificed at that time.

A change in the appearance of the focal process was detected in guinea pigs sacrificed one month after the beginning of the experiment. The lesions were now larger. Numerous granulomas appeared to have fused to form a single large granuloma (fig. 6). The constituent small granulomas were equal in size and appearance to those observed at 5 days and could be sharply delineated.

An area resembling hyaline necrosis began to appear in the center of the large granuloma at one month and was most prominent in the livers of guinea pigs permitted to survive for three months (fig. 7). The clear central areas seemed to contain dense masses of reticulum fibers with the azocarmine stain. A few polymorphonuclear leucocytes as well as dark staining cellular debris were scattered throughout the hyaline center. The remainder of the granuloma was unchanged in appearance from that which was seen earlier. No fibrosis could be demonstrated at the periphery of these lesions by the azocarmine stain. Langhan's giant cells were also recognized in the liver at this period.

Granulomas with areas resembling central hyaline necrosis were also evident in the livers of guinea pigs sacrificed 6 months after inoculation with the "Lynch" strain of *Br. abortus*. At this stage of the disease there were fewer cells involved in the focal process. Relatively few epithelioid and polymorphonuclear cells were scattered about in contrast to the compact layer of these cells seen at 3 months (fig. 8). This process was observed repeatedly in the livers of guinea pigs sacrificed after a similar period of infection due to other strains of brucella. Although casual inspection may suggest that this was due to a fortuitous arrangement of cells, the evidence clearly indicates that it is a specific process resulting from infection by brucella. Brucella have been isolated repeatedly from involved livers and the lesion has not been observed in uninfected control animals. Intermediate stages have been noticed between this lesion and the unmistakable granuloma. The epithelioid cells which were always present were definitely recognized, and their number though markedly reduced was considerable. The presence of scattered polymorphonuclears and their debris in the central areas was typical of the change which characterizes the known granuloma of brucellosis in guinea pigs.

When guinea pigs were sacrificed one year after inoculation with *Br. abortus*, their examination was characterized by the complete absence of specific histopathologic changes in serial sections of the liver and uniform lack of success in isolating the organisms.

Ten infected guinea pigs from the group of 24 were successfully rendered anergic by the weekly subcutaneous injections of heat-killed *Br. abortus*. Granulomas were found in the tissues of most of the anergic animals by the routine examination of 4 serial sections made from random blocks of the liver, kidney, bone marrow, and testicle. The results are summarized in table 1.

DISCUSSION

The events leading to the formation of the hepatic granuloma in experimental brucellosis due to *Br. abortus* begin

TABLE 1.—*Relationship of dermal hypersensitivity to formation of granuloma in experimental infections of* Br. abortus: *occurrence of granulomas in tissues of infected guinea pigs maintained in an anergic state by subcutaneous inoculations of increasing doses of heat-killed* Br. abortus *at weekly intervals.*

No. of Animal	Strain of *Br. abortus* used for infection	Duration of infection in months	Intradermal test with brucella antigen	Microscopic findings in 4 serial sections of liver, bone marrow, gonads, and kidney	Bacteriologic and serologic findings
4984	"Lynch"	1	–	One large granuloma in liver; no necrosis, scattered small granulomas.	*Br. abortus* isolated from spleen, kidney, and blood; agglutinins, 0.
4227	"Lynch"	1	–	Large granuloma in liver; no necrosis.	*Br. abortus* isolated from blood, bone marrow, and liver; agglutinins, 1:320.
4060	"Lynch"	1	–	No abnormality.	*Br. abortus* isolated from spleen; agglutinins, 1:160.
3760	"19"	1	–	Large granulomas with hyaline centers in liver.	*Br. abortus* isolated from spleen, liver, and blood; agglutinins, 1:320.
3772	"19"	1	–	No abnormality.	*Br. abortus* not recovered; agglutinins, 1:160.
4191	"19"	1	–	No abnormality.	*Br. abortus* cultured from lymph node and bone marrow; agglutinins, 1:2560.
4233	"Lynch"	3	–	Large granulomas in liver; no necrosis.	*Br. abortus* isolated from spleen; agglutinins, 1:5120.
4169	"19"	3	–	No abnormality.	*Br. abortus* cultured from kidney; agglutinins, 1:1280.
4068	"19"	3	–	One small granuloma in liver.	*Br. abortus* not recovered; agglutinins, 1:2560.
4151	"19"	3	–	Granulomas in bone marrow, liver, and kidney.	*Br. abortus* not recovered; agglutinins, 1:5120.

when brucella enter the general circulation. Many circulating organisms are removed from the plasma by polymorphonuclear leucocytes. Others are ingested by Kupffer's cells. Groups of polymorphonuclears containing brucella collect in the sinusoids of the liver and become mingled with Kupffer's cells. Adjacent Kupffer's cells, packed with brucella, also collect into groups at a somewhat later period, during which time the stray polymorphonuclears circulating in the sinusoids become greatly reduced in number. This appears to be the result of ingestion of parasitized polymorphonuclears by Kupffer's cells. The cellular aggregates gradually increase in size and form two types of granulomas. In one of these, little except epithelioid cells may be seen. These undoubtedly arise from the focal aggregations of Kupffer's cells. The other is composed of both polymorphonuclears and epithelioid cells and can be traced from the initial collection of polymorphonuclears and Kupffer's cells in the sinusoids. But few brucella are present in the cells of the developed granuloma.

Early granulomas then fuse with each other to form larger lesions. A small hyaline area may gradually appear in the center of these large lesions, but only rarely do they acquire a fibrous capsule. As the reaction in the tissues begins to subside, the cellular elements of the granuloma become reduced in number and may form a sparse collection about the hyaline center. After the infection terminates, no scar remains. The cells disperse and the small clear centers disappear.

It is to be emphasized that these studies pertain only to the hepatic granuloma in experimental brucellosis due to *Br. abortus* in mice and guinea pigs. The mechanism of abscess formation in infections due to *Br. suis* has not been fully clarified, although experiments indicate that the abscess is a direct outgrowth of the initial collection of polymorphonuclears in the hepatic sinusoids. Fibrous capsules are known to develop after the abscess becomes established, but there is no information con-

cerning the manner in which complete healing occurs. In an effort to produce healing, guinea pigs infected with *Br. suis* were treated with either aureomycin or a combination of streptomycin and sulfadiazine. Neither method was entirely successful, but there were noted two phenomena suggestive of early healing. One of these was characterized by calcification of the purulent center of an abscess and the other by a great increase of Langhans' giant cells in the granulomatous wall of an abscess.

The nonsuppurative granuloma represents a successful defense of the host against the invasion of brucella. The cells which enter into the formation of the granuloma are those which remove the brucella from the circulating body fluids. As the focal collections of phagocytic cells increase in size, the organisms drop out of sight from the circulation and become reduced in numbers in the cells themselves. It has been difficult to demonstrate the organisms in the cells of the granuloma after the 5th day. A predominance of mononuclear cells in the granulomas has been associated with a favorable course of the disease in the animals. The continued activity of polymorphonuclears with abscess formation is found in animals in poorer general condition.

The nonsuppurative granuloma of experimental brucellosis closely resembles the lesions of experimental tuberculosis. The hepatic tubercles are known to arise within the sinusoids from foci of polymorphonuclear and mononuclear cells, which phagocytose tubercle bacilli injected intravenously.[11,14] These cellular foci enlarge and the mononuclear phagocytes, which soon outnumber the polymorphonuclears, undergo transformation into epithelioid cells to form tubercles through a process which is apparently identical to that described in this report for the formation of the hepatic granuloma of brucellosis. The ultimate fate of the small focal lesions may also be the same in the 2 infections, as either process may subside by a resolution without scar formation.

The results summarized in table 1 imply that hypersensitivity is not essential for the granulomatous reaction of the tissues in experimental brucellosis and may be compared to evidence cited that the formation of the tubercle does not depend upon tuberculin sensitization.[9–11] In several respects, therefore, the focal cellular reactions of the tissues induced by two unrelated organisms have common features in their development as well as their morphologic appearance.

SUMMARY AND CONCLUSIONS

1. The basic granuloma, which is common to infections by all three species of brucella, has been traced in its development from small aggregates of cells in the sinusoids of the liver. The significance of the granuloma as a mechanism of defense and the functions of the individual component cells have been considered.

2. Observations are recorded which indicate that the development of the granuloma does not depend on the existence of hypersensitivity in experimental brucellosis.

3. Comparisons have been made between the properties of the granuloma of experimental brucellosis and the tubercle of experimental tuberculosis.

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HEMAGGLUTININ-INHIBITING PROPERTY OF DUCK PLASMA EXHIBITED IN AGGLUTINATION REACTIONS INVOLVING DUCK ERYTHROCYTES AND PLASMA OF CHICKS RECOVERED FROM LOPHURAE MALARIA

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The term sparing phenomenon originally implied plasma or serum activity in the role of protecting erythrocytes from destruction in the circulating blood of a host. The circumstances under which the phenomenon was observed[1–3] were as follows: Each of a group of chicks was injected intravenously with a known volume of duck plasma or serum, then with a known number of duck erythrocytes parasitized with *Plasmodium lophurae*. At the end of a variable interval of time, e.g., 1 hour, 5 hours, 1 day, or 2 days, it was found that there had been significantly less disappearance of parasitized duck erythrocytes from the blood of the chicks of this group than from another group injected with physiological salt solution and parasitized duck erythrocytes.

In this manner it was learned that the duck plasma spared (parasitized) duck cells from the full impact of the destructive forces to which foreign inclusions in the circulating blood of young chicks might otherwise be subjected. It has been shown[2] that the sparing phenomenon cannot be interpreted as a nonspecific protein effect, an illusion due to hemolysis of chick cells by duck plasma or serum, blockading (or blocking) of the macrophage system, a general intoxication of the chick produced by an heterologous plasma, an effect of the anticoagulant, or a reduction in the lag-phase of the reproduction of a parasite not yet fully adapted to the new host species.

Though it seemed that almost every objection that could be leveled against the reality of the sparing effect had been answered, it was suggested to us that an investigation of the agglutinating properties of the serums might prove enlightening on the grounds that, while weak agglutinins favor phagocytosis, strong ones inhibit it by forming such large clumps that they cannot be phagocytosed. Reference was made to the finding of Knisely et al[4] that in knowlesi malaria in monkeys parasitized "coated" red cells or small clumps of them are instantly ingested on contact with the sinusoid-lining von Kupffer cells, while large masses of "coated" agglutinated parasitized and unparasitized cells not only resist passage through the liver sinusoids but are too

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The authors acknowledge with gratitude the suggestion of Dr. W. H. Taliaferro of the University of Chicago that the agglutinating properties of the serums be investigated.

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large to enter phagocytes readily. Although, on the one hand, no special study had been made of the extent of randomization of the distribution of the parasitized duck cells in the stained smears of the blood of the chick recipients of duck plasma, it appeared, on the other hand, that such a study was not indicated because of the lack of clumps of parasitized red cells in smears of the blood of chicks in which the sparing phenomenon was occurring. It was felt, nevertheless, that there was sufficient reason why an investigation should be made of the agglutinating properties of duck and chick plasmas respecting duck erythrocytes. The present paper constitutes the first report of what the authors believe could prove to be a manifestation of the sparing phenomenon in vitro.

TABLE 1.—*Agglutinating titers and counts of parasitized cells (P.C.) for chicks injected with washed parasitized duck cells.*

	Chick No.	Agglut. titer	% P.C. 2 days	% P.C. 5 days	% P.C. 6 to 7 days
Group 1	1	0	1.5	24.5	10.0
age,	2	0	+*	0.2	2.2
16 days	3	0	5.5	57.5	1.7
	4	0	0.1	8.9	15.0
	5	0	4.3	12.3	0.0
	6	0	2.2	25.0	0.1
	7	0	5.6	48.0	13.7
	8	0	2.0	47.0	21.0
	9	+	0.0	+*	0.6
	10	0	0.9	18.5	50.5
Group 2	11	0	8.0	57.0	47.5
age,	12	0	6.4	50.5	36.5
20 days	13	0	1.2	12.3	34.0
17 to 22	14	0–+	3.0	22.5	39.5
injected	15	0	4.0	33.0	5.6
with	16	0	5.8	10.8	0.1
fraction C	17	0–+	0.1	4.3	6.0
	18	0–+	3.6	4.5	0.1
	19	0	0.3	2.6	3.2
	20	+	1.8	7.0	3.8
	21	0	7.3	44.5	24.0
	22	0	4.6	49.5	41.5
Group 3	23	0–+	0.1	0.5	0.3
age,	24	+	0.0	0.0	0.0
48 days	25	0–+	0.1	1.0	6.5
	26	++	0.0	0.0	0.0
	27	0	3.3	56.5	39.5
	28	+++	0.0	0.0	0.0
	29	0	2.0	49.0	28.5

* Less than 0.05%.

MATERIALS AND METHODS

The chicks employed were New Hampshire Reds from a hatchery, and the strain of *Pl. lophurae* was one carried on for several years in this laboratory by blood-passaging through ducks. From a vein of each chick listed in table 1 about 2 cc of blood were drawn into a syringe containing a few small crystals of sodium citrate, and after mixing, this blood was transferred to a conical centrifuge tube and centrifuged. Usually 0.5 cc of the supernatant plasma was transferred to a 75 mm×10 mm test tube, then 0.5 cc of the duck cell suspension was added, and the mixture shaken for 15 minutes. In the case of these groups it was necessary to transfer drops from the tubes onto glass slides for observation under the microscope to ascertain if agglutination was occurring. The chicks of each group were injected with uniform doses of parasitized duck cells per 100 g of body weight, as in the previous experiments.[1,2] The percents of parasitized red cells appearing in the table are based on readings of slides of chick blood made at the stated intervals and stained in Giemsa.

The chick plasma utilized in the tests for sparing effect in vitro (tables 2 and 3) was from birds of 40 days to 10 weeks of age which had recovered from lophurae malaria one to 7 weeks previously. Plasma of uninfected fowls was not tested. The anticoagulant was sodium citrate. It should be stated at this point that the effect of citrate in the strength utilized as anticoagulant was carefully tested for agglutinating and agglutination-inhibiting effect, without positive results.

In each 70 mm×10 mm test tube, 0.6 cc of the chick plasma was mixed with 0.3 cc of duck plasma, duck plasma dilution, or fraction of duck plasma being tested. Then 0.3 cc of duck red cell suspension was added to each tube, which was immediately mixed, and the whole assemblage of tubes shaken vigorously for 15 minutes. Readings were recorded at the time the most characteristic differences were noted, which in some instances was 25 minutes after shaking was completed, in other instances an hour or more. The red cell suspension represented, in most cases, a 1:8 dilution of the original red cell concentration in the blood of the duck, only the plasma had been removed and the cells washed three times in physiological salt solution as formerly described.[2]

RESULTS

Three age groups totaling 29 normal chicks were employed in tests to determine whether their bloods contained natural agglutinins to (parasitized) duck red cells and whether the birds with the strongest agglutinins exhibited the most

TABLE 2.—*Agglutinating titers at 1½ hours of mixtures of immune chick plasma and fraction C of immune blood when parasitized duck erythrocytes were used as antigen.*

1/2 chick plasma	1/4 frac. C	±	1/4 chick plasma	1/4 frac. C	0
1/2 chick plasma	1/8 frac. C	+	1/4 chick plasma	1/8 frac. C	±
1/2 chick plasma	1/16 frac. C	++	1/4 chick plasma	1/16 frac. C	+
1/2 chick plasma	1/32 frac. C	+++	1/4 chick plasma	1/32 frac. C	++
1/2 chick plasma	no frac. C	++++	1/4 chick plasma	no frac. C	+++
no chick plasma	1/2 frac. C	0	no chick plasma	1/8 frac. C	0
no chick plasma	1/4 frac. C	0	saline solution		0

rapid or the slowest rate of disappearance of infected duck cells from the circulating blood. The 29 birds were of the same hatching. Six birds, 17 to 22 in table 1, were injected one and one-half hours before the inoculation with the parasitized duck erythrocytes with 1 cc of "fraction C" of immune duck plasma which has been shown to contain the bulk of the sparing factor.[3] The results are summarized in table 1.

Only one of 10 16-day-old birds and one of 12 20-day-old birds exhibited definite agglutinins for the parasitized duck cells, though there were three others that exhibited feeble agglutinins. Of 7 older birds of group 3, however, three definitely agglutinated the duck cells in varying degrees, two showed feeble agglutinins, while only two failed to agglutinate the duck cells.

After the bloods were drawn for the agglutination tests, the groups were injected with erythrocytes parasitized with *P. lophurae* from the same duck as the cells used in the agglutination tests. While the doses were graded so as to be uniform according to body weights within the groups, no comparisons of the data should be made between groups. The 2-day counts represent a measure of the survival of duck erythrocytes in the bloods of the various chicks. It will be noted that the only negative smear in group 1 was from bird 9, the only one whose blood exhibited agglutinating properties for the duck erythrocytes employed. The other counts on that day are extremely variable, ranging from less than 0.05% to 5.6%. In group 2 bird 20, which was the only one exhibiting definite agglutinins, had a low count, but not the lowest. The same is true of birds 14, 17, and 18 all with feeble agglutinins. Birds 11, 12, 15, 16, 21, and 22, constituting half of the group, did not agglutinate the duck cells and had the highest counts of all, ranging from 4 to 8%. In a general way, then, the birds exhibiting the strongest agglutinins developed the lightest infections, but the correlation is imperfect. It appears that the sparing effect of the injected "fraction C" might have been responsible for the presence of parasitized cells in the smear of bird 20. The three birds of group 3 (24, 26, and 28) which exhibited definite agglutinating properties yielded negative smears on day 2. The two birds with feeble agglutinating properties 23 and 25, had very low counts on the same day, while the two which did not agglutinate the duck cells, 27 and 29, had the highest counts.

The protocols constituting tables 2 and 3 are representatives of those of a much larger series of experiments, all of which support the same conclusions. The chick plasma of table 2 was from chicks 23 and 25 of table 1, taken several weeks after the parasite could no longer be found in the blood. It should be stated at this point that the agglutinating property of the chick plasmas was confirmed before the tests were made, because it is obviously impossible to demonstrate hemagglutinin-inhibiting properties of duck plasma or plasma fractions unless the chick plasma does

agglutinate the duck cells being employed. The plasmas of a number of the 29 chicks on hand agglutinated the duck cells only very feebly or not at all, and for this reason were unsuited for the tests. Fraction C was the protein precipitated from the pooled plasma of 8 immune ducks by 33.3% to 50% saturation with ammonium sulphate.[3] The agglutinating systems consisted of 0.6 cc chick plasma or dilution, 0.3 cc fraction C or dilution, and 0.3 cc of 1:8 parasitized duck red cell suspension.

whether normal duck plasma could be substituted for the immune plasma, and whether the amboceptor in chick plasma heated at 56 C for 35 minutes functioned the same as the unheated plasma which presumably contained complement. The arrays of tubes for the 4 series with immune duck plasma and the series with normal duck plasma were almost indistinguishable. These results, all of which were repeated later, showed that the normal duck erythrocytes functioned as well as the parasitized erythrocytes,

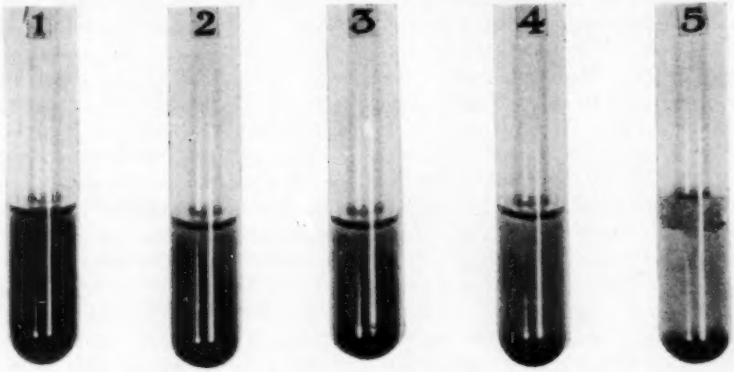

FIG. 1.—Array of tubes 1 to 5 of table 2 showing graded ascending series of precipitated red cells from left to right and of red cell concentration in supernatants from right to left.

The agglutinating titers (table 2) in the various tubes increased as the concentration of fraction 3 decreased (table 2 and fig. 1) when the systems were one-half chick plasma. The same held true when they were one-fourth chick plasma. Neither physiological salt solution nor any of the dilutions of fraction C agglutinated the infected duck cells. The role of fraction C as an inhibitor of hemagglutination is clear.

In table 3 are recorded the results of the first test to determine whether, in the agglutination system, nonparasitized erythrocytes from a normal (i.e., not previously infected) duck were as suitable antigen as the parasitized ones, that the heated chick plasma served as well as the unheated as the source of amboceptor, and that normal duck plasma could inhibit agglutination of parasitized duck cells as well as that from the immune duck. The one recording for nonparasitized erythrocytes and normal duck plasma showed that this combination operated the same as the others. Later tests with this combination and double dilutions of the duck plasma yielded arrays as typical of the sparing phenomenon as those with parasitized duck cells and immune plasma. It should be stated here that subsequent tests with both chick and duck plasmas heated at 56 C for either 30 minutes or

40 minutes yielded the same results as when either one or neither was heated.

As is shown in table 3, one-fourth and one-half strength duck plasma dilutions did not agglutinate either the parasitized or non-parasitized duck erythrocytes. Whether the same results would be obtained under all conditions cannot be stated at this time.

Other tests were made to determine whether the sparing factor was adsorbable on either immune or parasitized duck cells. The washed and centrifuged red cells from a volume of duck blood were mixed with the same volume of unheated duck plasma in some tests, with the same volume of duck plasma diluted (1:2) with physiological salt solution in others. The mixtures were pipetted into 20 cc screw-cap test tubes and hand agitated from 40 minutes to one hour. The cells were centrifuged down and the supernatant drawn off for use as adsorbed duck plasma. In the largest test, utilizing parasitized duck cells and the pooled plasma from two immune ducks and two normal ducks, were three series of tubes, one with the plasma of chick 23, one with the plasma of chick 25, and one with the pooled plasma of these two chicks. In each series was an array with nonadsorbed cells and one with adsorbed cells. The 6 arrays, each consisting of 6 tubes with total duck plasma contents of 1/4, 1/8, 1/16, 1/32, 1/64, and 0, respectively, presented almost perfectly graduated ascending series of inhibition from right to left. This experiment with parasitized cells and others with nonparasitized cells yielded no indications of notable adsorption of the sparing factor. In contrast, it should be stated that it was very easy to adsorb out the amboceptor (hemagglutinin) from the chick plasma by the above method, with practically all of it removed after one treatment.

TABLE 3.—*Agglutinating titers when comparisons are made of heated and nonheated chick plasma, parasitized and nonparasitized duck erythrocytes, and immune and normal duck plasma.*

Mixture		Parasitized RBC		Nonparasitized RBC	
		Unheated chick plasma	Heated chick plasma	Unheated chick plasma	Heated chick plasma
Immune duck plasma					
1/2 chick plasma	1/4 duck plasma	±	±	±	±
1/2 chick plasma	1/8 duck plasma	+	+	+	+
1/2 chick plasma	1/16 duck plasma	++	++	++	++
1/2 chick plasma	1/32 duck plasma	+++	+++	+++	+++
1/2 chick plasma	no duck plasma	++++	++++	++++	++++
no chick plasma	1/4 duck plasma	0	0	0	0
no chick plasma	1/2 duck plasma	0	0	0	0
saline solution		0	0	0	0
Normal duck plasma					
1/2 chick plasma	1/4 duck plasma		±		±
1/2 chick plasma	1/8 duck plasma		+		
1/2 chick plasma	1/16 duck plasma		++		
1/2 chick plasma	1/32 duck plasma		+++		
no chick plasma	1/4 duck plasma		0		
no chick plasma	1/2 duck plasma		0		

If the hemagglutinin-inhibiting principle is not adsorbed on the duck red cell, does it combine with the amboceptor in the chick plasma? When the chick plasma is mixed with the duck plasma no precipitate forms, not even clouding, if both plasmas are perfectly clear to begin with. When simple haptenes combine with antibody, however, no precipitate may be formed (Marrack[5]). The elucidation of the dual problem of whether the inhibition of the hemagglutinin is due to combination of a haptene-like substance with the antibody, and whether the haptene-like material is lipoid or

5. Marrack, J. R. 1938, Med. Research Council Sp. Rep. Ser. no. 230: 1–194.

protein in nature will require considerable further investigation.

It is to be noted in figure 1 that the red cell density of the supernatant in tube 4 is considerably greater than that in tube 5. In the experiments described above no greater dilutions of the duck plasma than 1:32 and 1:64 were tested, but in two other experiments in which a special point was made of determining the extreme sparing potency of the duck plasma, dilutions of 1:128 (unheated plasma) and 1:288 (heated plasma) yielded perceptible inhibition.

DISCUSSION

It is the major contribution of the present paper that duck plasma contains a substance that can inhibit the agglutination of duck cells by chick plasma in vitro. It is believed by the authors that the hemagglutinin-inhibiting phenomenon observed in vitro is a manifestation of the mechanism that produces the sparing phenomenon in vivo and duplicates the latter so far as obvious limitations permit. Even if the identity of the two phenomena were experimentally proved, to state that the findings in vitro confirm the observations in vivo would still be an unjustifiable exaggeration, for what is observed in the test tube is "sparing" of the duck erythrocytes from agglutination by chick plasma possessing hemagglutinin for duck cells. The process producing the disappearance of the duck cells from the circulating blood of the chick is considerably more complicated, involving opsonin and phagocytosis, and possibly hemolysis, as well.

The tests with heated chick plasma and unheated duck plasma and heated chick plasma and heated duck plasma eliminate the need for further consideration of the possible role of complement in the reaction. For the same reason, and because hemagglutinin-inhibition was obtained when fresh chick and duck plasmas were used, anticomplementary action can also be ruled out. These tests also demonstrated the thermostability of the inhibiting factor as well as of the hemagglutinin. It has been previously demonstrated by us in two series of tests in chick (unpublished) that heating the duck plasma at 65 C for 30 minutes did not significantly damage the sparing factor.

Although apparently complete adsorption of the hemagglutinin from the duck plasma with washed duck cells was easy to accomplish, adsorption with washed duck cells removed practically none of the hemagglutinin-inhibiting factor. This property of nonadsorbability places it in a different category from the incomplete antibody of Race,[6] the monovalent blocking antibody of Wiener,[7] both noted in the serums of certain individuals sensitive to the Rh factor, and the antibody of Levine and Gilmore[8] from mononucleosis patients which prevented agglutination of sheep erythrocytes. In each of these cases the principle that inhibited agglutination formed a coating, or was adsorbed, on erythrocytes. The recent demonstrations by Griffitts[9] of an agglutinin-blocking phenomenon in serums from cases of human brucellosis and by Cox and Kutner[10] in bovine serums concern cases wherein bacterial cells play the role of antigen. Griffitts found that heating the human serums at 56 C for 15 minutes usually eliminated its blocking property.

The minimum amount of duck plasma

6. Race, R. R. 1944, Nature, **153**: 771–772.
7. Wiener, A. S. 1944, Proc. Soc. Exper. Biol. & Med. **56**: 173–176.
8. Levine, P. and Gilmore, E. L. 1945, Science, **101**: 411–412.
9. Griffitts, J. J. 1947, Pub. Health Rep. **62**: 865–874.
10. Cox, C. D. and Kutner, L. J. 1950, Science, **111**: 545–546.

required to produce hemagglutinin-inhibition, which in one case was 1/128 of the total volume and in another case 1/288, indicates that the inhibiting factor is by no means either a negligible or an elusive component of plasma. In another report it has been shown that the bulk of the factor that produces sparing in vivo is in the protein fraction of duck plasma precipitated in the zone between 33.4% and 50% saturation with ammonium sulphate, but there is no reason for believing that all of this fraction is capable of playing a sparing role.

It was known[1,2] that the sparing phenomenon could be produced in chicks with either normal or immune duck plasma. It is now shown that either normal or immune duck plasma is operative in inhibiting the hemagglutinins in vitro. It appears that both antibody and sparing factor are basic functional components of the normal plasma.

Parasitized cells were required in the chick experiments because there was no other means of labeling the injected duck cells so that they might be positively identified. It was clearly recognized[2] that in such tests the fate of nonparasitized cells was not being determined. In the present tests nonparasitized cells of both normal and immune ducks sufficed as well as parasitized cells for antigen. The dual implication is that nonparasitized duck cells would also be spared in the chick under circumstances that spared the parasitized cells, and that the only advantage derived from the use of the parasitized cells was, as noted, labeling for recognition under the microscope.

The observation that nonparasitized duck cells as well as the parasitized cells were subject to hemagglutination by immune chick plasma recalls certain findings by Taliaferro and Cannon,[11] Taliaferro and Mulligan,[12] and Zuckerman.[13] Taliaferro and collaborators observed phagocytosis of uninfected red cells by macrophages just before or during the crisis in *Plasmodium brasilianum* infections in Panamanian monkeys and in *Plasmodium cynomolgi* and *Plasmodium knowlesi* infections in *Silenus rhesus*. Zuckerman, who made in vitro studies, reported that macrophages in serum from chickens hyperimmunized to *P. lophurae* and *Plasmodium gallinaceum* tended to ingest whole infected red cells as well as whole malarial parasites and whole parasitized red cells.

The success in producing the inhibiting phenomenon in vitro almost completely overshadows the results of the original design of the experiment—to test the possibility that the sparing phenomenon in vivo is attributable to excessive clumping of parasitized duck cells in the chick and failure of macrophages to phagocytose the aggregates. It has been stated that no such large aggregates have ever been observed in blood smears of chicks experiencing the sparing phenomenon. The results of tests appearing in table 1 furnish no case of the sparing phenomenon in birds with the higher hemagglutinin titers. In fact, the higher hemagglutinin titers attend rapid disappearance of injected duck cells.

CONCLUSIONS

1. What may prove to be the in vitro counterpart of the sparing phenomenon, viz., inhibition of hemagglutinins for parasitized duck erythrocytes in the plasma of chicks recovered from lophurae malaria, has been reproduced.

2. It appears to be a matter of indifference whether the antigen in the agglutination system is parasitized erythrocytes or nonparasitized erythrocytes

11. Taliaferro, W. H. and Cannon, P. R. 1936, J. Infect. Dis. **59**: 72–125.

12. Taliaferro, W. H. and Mulligan, H. W. 1937, Indian J. M. Research Mem. no. **29**: 1–138.

13. Zuckerman, A. 1945. J. Infect. Dis. **77**: 28–59.

from either normal or immune ducks.

3. The plasma of normal ducks appears to function in the system as the carrier of the hemagglutinin-inhibiting substance as well as the immune plasma.

4. The possibility of either complement fixation inhibition or anticomplementary action operating in the hemagglutinin-inhibiting phenomenon is excluded, because heating either chick or duck plasma or both at 56 C for 30 to 40 minutes does not inactivate the agent that produces the effect.

5. The hemagglutinin-inhibiting component of duck plasma is nonadsorbable on uninfected or infected duck erythrocytes.

6. The sparing phenomenon is not the result of the creation, by a super-abundance of hemagglutinin, of such large concentrations of erythrocytes that they cannot be phagocytosed.

7. The cause of relapse in malaria should be sought in the area of the interplay of sparing and so-called protective (i.e., hemagglutinin, opsonin, hemolysin) factors.

INEFFECTIVENESS OF CORTISONE THERAPY IN MICE INFECTED WITH JAPANESE B ENCEPHALITIS AND THE ADVERSE EFFECT OF HIGH DOSES

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Endocrine substances are among the presumptive agents of host constitution, and some of them have been shown to alter resistance to virus[1,2] or to change intercellular conditions affecting the spread of viral particles.[3] Adrenocortical hormones deter the spreading of injected particulate matter under certain experimental conditions,[4,5] but little has been reported on their use in viral infections. Cortisone and adrenocorticotrophic hormone (ACTH) were found ineffective against rickettsial infection in mice,[6] and there are clinical indications that ACTH is ineffective against poliomyelitis[7] and herpes zoster.[8] Since adrenocortical extract (ACE) has been reported to increase resistance of guinea pigs to *Clostridium welchii*[9,10] and that of mice to pneumococcal infection,[11,12] it appeared worthwhile to study the influence of adrenocortical hormones on the course of experimental viral infection. Japanese B encephalitis, parenterally induced, was considered a good test infection because of its long latent period and regular course in mice.

METHODS AND MATERIALS

Three-week-old male white Swiss mice were inoculated with the virus intraperitoneally or subcutaneously in the lower right abdomen. The Okinawa strain[13] of Japanese B encephalitis virus in the 7th intracerebral mouse passage was used. Brains of mice showing typical symptoms of the disease were removed and ground with alundum, and a suspension was prepared with 10% inactivated normal rabbit serum. The crude suspension was centrifuged for 10 minutes at 3000 r.p.m. and the supernatant removed for use in appropriate dilutions. The intracerebral titer of the suspension was determined for each test, using 5 mice for each decimal dilution. The LD_{50} is expressed as the exponent of the reciprocal of the LD_{50} dilution as determined by the method of Reed and Muench. Symptoms of the disease (paralysis, twitching of vibrissae, unsteady gait, bulging eyes) appeared in many mice about the 5th day, though occasionally as late as the second or third week, with death following in a few days. Deaths rarely occurred after the third week, and experiments were therefore brought to close at 21 days.

Treatments consisted of subcutaneous injections of hormone on the side opposite the site of introduction of the virus, once daily as a rule, from the day before infection up to the 9th day of

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TABLE 1.—*Mortality rates in 3-week-old male Swiss mice infected parenterally with Japanese B encephalitis and treated with adrenal cortical extract (ACE), cortisone, or testosterone for 10 days.*

Exp. no.	Virus* IC Titer (LD_{50})	Dilution and route	Treatment† & substance	Amount	No. of mice		Mortality ratio % exp.: % controls	*P* value
					Total	Dead		
1	8.7	2 IP	ACE	0.4 to 0.8 ml	50	35	0.93	>0.99
		2 IP	saline	0.4 to 0.8 ml	48	36		
		1 SC	ACE	0.4 to 0.8 ml	50	24	1.04	>0.99
		1 SC	saline	0.4 to 0.8 ml	50	23		
2	8.0	3 IP	Cortisone	50 to 150 μg	50	34	1.10	>0.50
		3 IP	saline	0.1 ml	50	31		
3	8.4	2 IP	Cortisone	250 μg	48	42	1.15	>0.20
		2 IP	saline	0.1 ml	50	38		
		1 SC	Cortisone	250 μg	50	42	1.50	<0.01
		1 SC	saline	0.1 ml	50	28		
4	9.0	2 IP	Cortisone	250 μg	50	46	1.10	>0.20
		2 IP	saline	0.1 ml	50	41		
		1 SC	Cortisone	250 μg	50	34	1.62	0.02
		1 SC	saline	0.1 ml	50	21		
		(No virus)	Cortisone	250 μg	30	0		
5	9.2	3 IP	Test. prop.	250 μg	50	35	0.83	>0.10
		3 IP	sesame oil	0.05 ml	50	42		
		2 SC	Test. prop.	250 μg	50	32	0.86	>0.30
		2 SC	sesame oil	0.05 ml	50	37		

* 0.3 ml of virus suspension per injection; titer of inoculum expressed as exponent of the reciprocal of the dilution.
† Treatment began one day before infection and continued for 9 additional days, discontinued for final 12 days.

infection (ACE was given twice daily from the 5th day on, and in one experiment cortisone dosage was similarly varied). Adrenocortical extract (Upjohn), cortisone (Merck) and testosterone propionate (Rare Chemicals) were used, the first two with saline ("Vacoliter") for control injections, the last with sesame oil. Dilutions of cortisone and testosterone were made with saline solution and sesame oil, respectively. All injection procedures were randomized for even dissemination of substances and equal handling of mice.

RESULTS

The administration of ACE with an activity equivalence of about 40 to 80 μg of cortisone daily had no effect upon the course of intraperitoneal or subcutaneous infection (table 1, exp. 1). Similarly cortisone, at 50 to 150 μg daily, did not affect the outcome of intraperitoneal infection (exp. 2). In a group injected with 250 μg of cortisone daily, however, the mortality rate was increased approximately 50% over that of the control group when the injection of virus was subcutaneous, while there was no significant difference when the injection was intraperitoneal (exp. 3). In order to verify these results the experiment was repeated with minor variations of procedure, and almost identical results were obtained: 60% more deaths in cortisone-treated mice than in control mice with subcutaneous infection, and insignificantly more in the cortisone-treated group with intraperitoneal infection (exp. 4). There were no deaths among uninfected cortisone-treated controls.

In order to determine whether the adverse effect associated with the high dose of cortisone might be common to steroid hormones, a comparable experiment was set up with testosterone propionate. There was no significant difference between the group injected with this hormone and the control group injected with sesame oil (exp. 5).

Groups of immature male mice were injected with different doses of cortisone for 10 days, weighed daily, and observed for an additional period of 11 days. No deaths occurred among 10 animals from the same weaning pool and

TABLE 2.—*Effects of 10 day-treatment with cortisone on 3-week-old male mice.*

Group (10 mice)	Daily dosage: μg/mouse	mg/kg (av)	Av initial body weight	Weight increase during treatment	Days on which deaths occurred	Comment
1	0	0	12.3	48%		Normal controls
2	40	3.6	11.0	38%	2, 11	Puniest mice, probably a poor test group; 2 dead
3	100	7.8	12.9	44%		Normal growth curve
4	250	20.0	12.5	37%		Normal growth
5	250	21.2	11.8	14%		Growth retarded
6	250	20.0	12.3	11%		Growth retarded
7	625	51.8	12.1	16%	6, 6, 8	Growth retarded; 3 dead
8	1560	132.3	11.8	17%	2, 8, 9, 10, 10, 12,	Growth retarded; 6 dead
			M = 12.1			

The above indicates that doses of cortisone up to 250 μg/mouse/day are unlikely to be lethal in the 12-gram, 3-week-old male, but that growth may be retarded at the latter dosage. The LD_{50} is approximately 1.1 mg (92 mg/kg). All groups except the 5th and 6th came from the same pool of animals as those of exp. 4 (table 1) and were treated concomitantly and with the same hormone preparations.

injected with the same amount of cortisone as in experiment 4; their growth was essentially normal (table 2, group 4). All mice in two similar groups observed later at this dosage (250 μg) also survived in apparent good health, but their growth was retarded during treatment (groups 5 and 6). The next higher dose, 650 μg, retarded growth and resulted in three deaths (group 7). The highest dose, 1560 μg, resulted in 6 deaths, a rate comparable to that reported by Antopol[14] in similar young mice at a similar dosage. It is evident that 625 and 1560 μg daily (50 to 130 mg/kg) represent frank overdosage in normal uninfected mice, while 250 μg (20 mg/kg) represents high dosage perhaps at the borderline of "overdosage."

DISCUSSION

The administration of adrenocortical substances (in cortisone or ACE) at 40 to 100 μg daily did not affect the mortality rate of mice infected with the virus. At 250 μg (about 20 mg/kg) the mortality rate was increased, and this change may be attributed to a combined effect of hormone and virus, since no deaths occurred among uninfected controls treated with this dose of cortisone. While 250 μg daily was not fatal in uninfected animals, it was sufficient to inhibit body growth.

The evaluation of dosage is important, since, as recently noted by Sayers,[15] there is no fixed amount of cortical hormone that represents a physiological dose, and the needs of tissues vary under different conditions. It has been assumed that in some types of bacterial infection the need for adrenocortical hormone is high relative to endogenous capacity, so that treatment with ACE is beneficial.[9,10,12] In the present experiments it may be thought that the virus does not appreciably change the need for hormone up to the time that the nervous system has been attacked, and that therefore moderate doses of hormone do not affect the course of infection. These animals, in fact, may maintain normal blood cortical hormone values through pituitary adjustment. On the other hand the higher dose (250 μg) may be thought to inhibit the pituitary to the limit, flood the tissues with cortisone, and produce metabolic changes unfavorable to resistance to the virus as well as to body growth.

It appears possible, on the basis of very limited information, that the extent of involvement of an adrenocortical factor in resistance differs widely from one type of infection to another. Thus in

14. Antopol, W. 1950, Proc. Soc. Exper. Biol. & Med. **73**: 262–265.

15. Sayers, G. 1950, Physiol. Rev. **30**: 241–320.

some instances there may be an increase in relative need and hence some value in hormone therapy, while in others the need is unchanged and there is no benefit from such therapy. The bacterial and viral infections which have been studied may differ in this respect, according to differences in the biological properties of their respective agents.

SUMMARY AND CONCLUSIONS

Moderate doses of adrenocortical extract or cortisone had no effect on Japanese B encephalitis virus infection in mice. Doses of cortisone that inhibited growth in uninfected mice also increased the mortality rate in infected mice. This was not a general steroid effect, since testosterone in equivalently high dosage had no adverse effect. It was concluded that relative insufficiency of adrenocortical hormone was either not present in the virus-infected mice, or, if present, did not appreciably influence the course of infection, so that supportive therapy could not affect the outcome. The adverse effect of the high dose of cortisone on resistance was attributed to the metabolic results of overdosage.